Chemical Science Review and Letters ISSN 2278-6783

Research Article

Compounds from Natural Sources as Potential Anti-Cancer Agents: An *In Vitro* Study

Ashrini BS* and Varalakshmi KN

Department of Biotechnology, Centre for Post-Graduate Studies, Jain University, Jayanagar, Bangalore, Karnataka State, India

Abstract

Cancer, also termed as malignancy, is the uncontrolled division and spread of abnormal cells. The primary modalities of cancer treatment are surgery, chemotherapy and radiotherapy, but due to poor survival rates and inefficient cancer control rates, new therapeutic strategies are required for effective treatment of cancer. A great number of anticancer compounds are natural products or their derivatives, mainly produced by microorganisms. With respect to this, the aim of the present study was to isolate and screen microorganisms from unexplored ecological niches for potential anti-cancer compounds. *In vitro* anticancer activity was carried out against human cervical cancer cell line, HeLa by MTT assay. Partial purification was performed by TLC and bioactive fraction was identified as per the standard protocols. One of the fungal isolates, isolated from a soil source, exhibited promising anticancer activity against HeLa cells.

Keywords: Cancer, anticancer compounds, HeLa cells, Fungi

***Correspondence**

Author: Ashrini

Email: ashrinisuresh@gmail.com

Introduction

Cancer also termed as malignancy is the uncontrolled division and spread of abnormal cells. All cancers involve the malfunction of genes that control cell growth, division and death. However, most of the genetic abnormalities that affect risk of cancer are not hereditary, but result from various damages to the genes caused by mutations that occur throughout a person's lifetime [1]. The primary modalities of cancer treatment are surgery, chemotherapy and radiotherapy, but in a few cancers such as mesothelioma, certain cases of breast cancer, multimodal treatment involving surgery, chemotherapy and radiotherapy has failed to yield efficient survival rates [2, 3]. Due to poor survival rates and inefficient cancer control rates, new therapeutic strategies are required more than ever for effective treatment of cancer. New strategies involve the use of novel compounds isolated from natural sources. Natural sources like plants and microbes have provided many bioactive compounds with anti-hyperglycemic, antibacterial, antifungal, antioxidative and anticancer activities [4, 5, 6].

Anticancer activity is the effect of natural, synthetic or biological and chemical agents to reverse, suppress or prevent the carcinogenic progression [7]. Microbes as natural sources have long history of use in the treatment of cancers with promising anticancer agents being approved by the FDA and few others in the clinical stage of testing [8]. In the recent years' filamentous fungi have gained much popularity because of their diverse secondary metabolite production, which find applications in various fields ranging from medical, industrial and agricultural industry [9, 10]. Among the filamentous fungi, *Penicillium* species occupy an important position. *Penicillium* belong to the genus of ascomycetes fungi which are of major importance in the field of food and drug production. Since the discovery of Penicillin [11], intensive studies have demonstrated the use of secondary metabolites produced by *Penicillium* sp. to be useful in production of various bioactive agents like antibiotics, anticancer agents which are being used in the treatment of various diseases [12, 13].

Current debate revolves around the lack of efficacy or unacceptable levels of toxicity caused by chemotherapeutic drugs which increases the demand for novel antitumor drugs active against untreatable tumors, with lesser side effects and more efficiency [14]. Considering this as the main aim we have screened a number of microbes from various soil sources for their anticancer activities. Among the different isolates, the fungi belonging to the *Penicillium* sp. was selected for the study.

Materials and Methods

Collection of Soil sample

Rich soil samples for the current study were collected from different locations in and around Agumbe forest, a part of the Western Ghats mountain range, India [13.50°N,75.09°E] in sterile autoclaved bags and were preserved at 4°C until further use.

Isolation and Identification

Isolation of microbes from the soil samples were carried out by serial dilution and spread plate techniques. The dilutions were made up in sterilized distilled water up to 10^{-4} dilutions. The serially diluted cultures were spread on Nutrient agar medium for bacteria and Czapek Dox agar for fungi. Following inoculation, the bacterial culture plates were incubated at 37°C for 72h in an incubator and the fungal plates were incubated at room temperature for 7 days. Pure cultures were obtained by subculturing individual colonies on to plates containing Nutrient agar and Czapek Dox agar for bacteria and fungi respectively.

Preliminary identification was carried out based on microscopic and cultural observations. Molecular identification on the promising fungi was performed by sequencing the conserved 18s ribosomal RNA.

Production of Secondary Metabolites

Based on the results of initial screening, fungus was selected for studies. The fungal isolate was grown for 7days at room temperature in Czapek dox broth in a 250ml conical flask prior to extraction. The fungal biomass was collected by filtering the media using a whatmans filter paper and was kept for drying in a hot air oven at 50°C overnight. The dried biomass was homogenized using mortar and pestle with methanol and centrifuged at 10,000 rpm for 10minutes.The supernatant was collected and left to evaporate at room temperature [15].

TLC Fractionation

TLC separation was performed using commercially available silica plates, which were activated at 110°C for 30minutes in a hot air oven prior to use. The solvent mixture for TLC fractionation was optimized by trial and error method, using a combination of polar and non-polar solvents. The extract dissolved in methanol was spotted one centimeter above the bottom end of the TLC plate and allowed to run till the solvent front reached two centimeters from the top end of the TLC plate. The plate was then dried and the spots were detected by exposing the TLC plate to UV light at 254nm and 365nm wavelengths. The Rf values of each band were obtained by determining the distance run by each spot from the origin. Bioactive fraction was obtained by conducting preparative TLC, where each spot was scraped carefully from the plates and dissolved in methanol. The fractions were separated from the silica particles by centrifuging at 10,000 rpm for 10minutes and each fraction was tested for cytotoxicity.

Screening for anticancer activity

The effect caused by the crude extracts of the two bacterial and fungal isolates along with the TLC purified bioactive fraction was tested on Human cervical cancer cell line (HeLa)by conducting MTT cell viability assay, trypan blue cell counting, DNA fragmentation assay, lactate dehydrogenase assay (LDH) and caspase activity assay.

MTT Assay

MTT [3- (4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] assay [16] was performed on treated and control HeLa cells. The cells were seeded in 96-well plates and incubated for 24h at 37°C in a 5% CO_2 incubator for cell adhesion. 1,10,50 and 100µg/ml concentrations of crude extract and 1,5,10 and 20µg/ml concentrations of TLC purified bioactive fractions were added in triplicates and incubated for 24hours and 24,48 and 72 hours respectively. After the incubation period, 100µl of MTT solution was added to each well and the plates were incubated in dark for 3hours at 37°C, after that 100µl of DMSO was added to each well. The absorbance was recorded at 540 nm using an ELISA plate reader. The percentage cell viability was determined using the following formula:

$$\text{Percentage viability (\%)} = (A_{540} \text{ of the test sample})/ (A_{540} \text{ of the control}) \times 100$$

Trypan Blue Cell Staining

Trypan blue cell staining assay was performed to determine the number of viable cells present in cell suspension of extract treated cells. The assay was conducted according to protocol mentioned in strober *et al* 2011[17]. The percentage viability of treated cells was calculated using the formula:

$$\text{Percentage viability (\%)} = (\text{Number of viable cells})/ (\text{Total number of cells}) \times 100$$

DNA fragmentation

To determine the extent of DNA fragmentation in treated cells, 400µL of 20µg/ml bioactive fraction was added to monolayer of HeLa cells grown in a $25cm^2$ tissue culture flask. After 24h of incubation at 37°C, the cells were harvested, lysed and the DNA was isolated using DNA isolation kit(HiMedia). The extracted DNA was electrophoresed using 0.8% agarose gel containing Ethidium Bromide.

LDH Assay

Cytotoxicity caused by the promising bioactive fraction was analyzed by measuring the activity of the cytosolic enzyme Lactate Dehydrogenase (LDH), which is released when the cells undergo lysis, was measured with a coupled enzymatic reaction. Extract treated cells were analyzed for LDH activity using CytoscanTM LDH Assay kit (G-Biosciences) as per the manufactures instructions. OD was measured at 490nm using a micro-plate reader and percentage cytotoxicity was determined.

Caspase Activity

CasPASE Apoptosis Colorimetric Assay Kit (G-Biosciences) was used to determine the effect of the bioactive fraction on the induction of apoptosis on HeLa cells, by measuring the activity of caspases as per the instruction manual. Optical density was recorded in an ELISA plate reader (500 nm) every 30 min, until significant differences in the readings were observed.

Results and Discussions

Isolation and Identification

Soil has remained an excellent source for a variety of microbes, ranging from eukaryotic bacteria to prokaryotic viruses and fungi. Out of the four microbial isolates of bacteria and fungi tested for their anticancer activity by MTT, the fungi (B Fungi) belonging to the genus *Penicillium* produced the highest cytotoxicity against human cervical cancer cell HeLa (**Figure 1**).

Preliminary identification was carried out based on morphological and colony characterization. The colonies were greenish white in color and produced a diffusible yellow pigment when grown on Czapek dox agar. Microscopic evaluation of the fungi showed a dense felt of conidiophores. Based on preliminary identification and morphological observations, the metabolite producing fungi was identified to be a member of the *Penicillium* species (**Figure 2**).

Molecular identification confirmed the isolate to belong to the genus *Penicillium* and the 18S ribosomal RNA gene sequence matched 98% with *Penicillium* sp. PSF42. Hence our promising isolate (B Fungi) was named as *Penicillium* sp. JUFP2.

Partial Purification by TLC

Following methanol extraction of the fungal biomass, the solvent was left to evaporate at room temperature to obtain 280mg of dried extract. For TLC separation of bioactive fractions, a combination of polar and non-polar solvent system was used,the highest separation and resolution was observed using the solvents dichloromethane : formic acid in the ratio 20:1, when the TLC plate was placed at 90° to the solvent chamber (**Figure 3**).

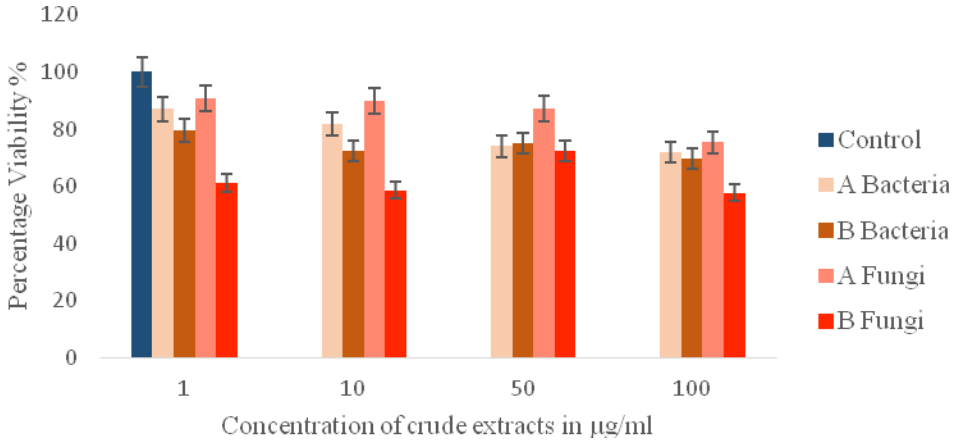

Figure 1 Effect of different concentrations of Bacterial and Fungal extracts on HeLa cell percent viability when treated for 24h

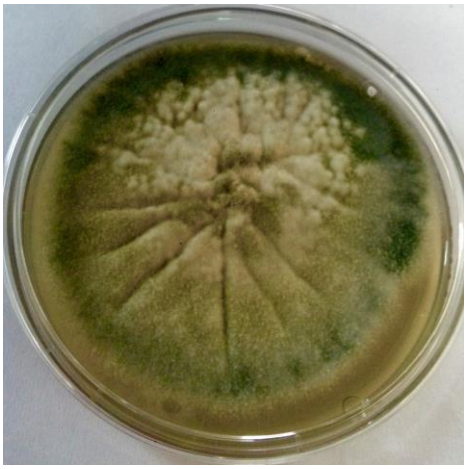

Figure 2 Pure culture of metabolite producing Fungi of *Penicillium* species on Czapek Dox agar

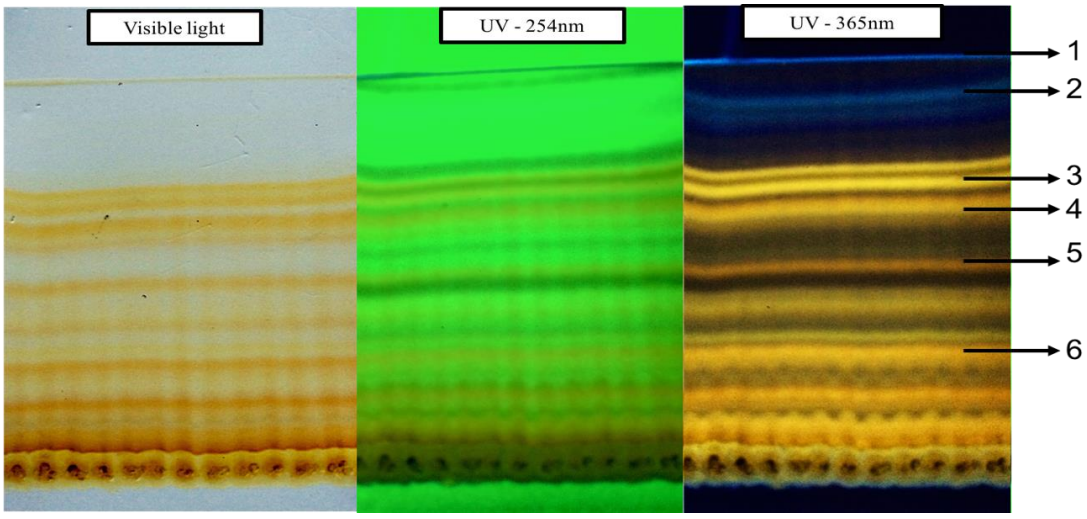

Figure 3 Thin-layer chromatography of separated fractions, under visible and ultraviolet light at 254 and365nm using solvent system of dichloromethane: formic acid in the ratio 20:1.1: Solvent Front and fraction 1.2: Fraction 2.3: Fraction 3.4: Fraction 4.5: Fraction 5 and 6: Fraction 6

Cytotoxic effects of Bioactive Fraction 2

When Hela cells were treated with the different fractions separated in TLC, Fraction 2 showed the best cytotoxic results out of all the tested fractions (Results not shown). The Bioactive fraction 2 was further tested for its anticancer and antiproliferative activity on the HeLa cells by treating them with this fraction for 24, 48, and 72 h. Fraction 2 exhibited the highest cytotoxic activity at 10 µg/mL (47.38%) and 20µg/mL (41.95%) concentrations after 72 h (**Figure 4**). From this it could be inferred that the inhibition on the viability of HeLa cells was dose- and time-dependent. As the concentration and time of exposure to the bioactive fraction increased, there was a decrease seen in the percentage viability of the HeLa cells. From the dose response curve the IC_{50} value was found to be 2.29µg/ml for this fraction.

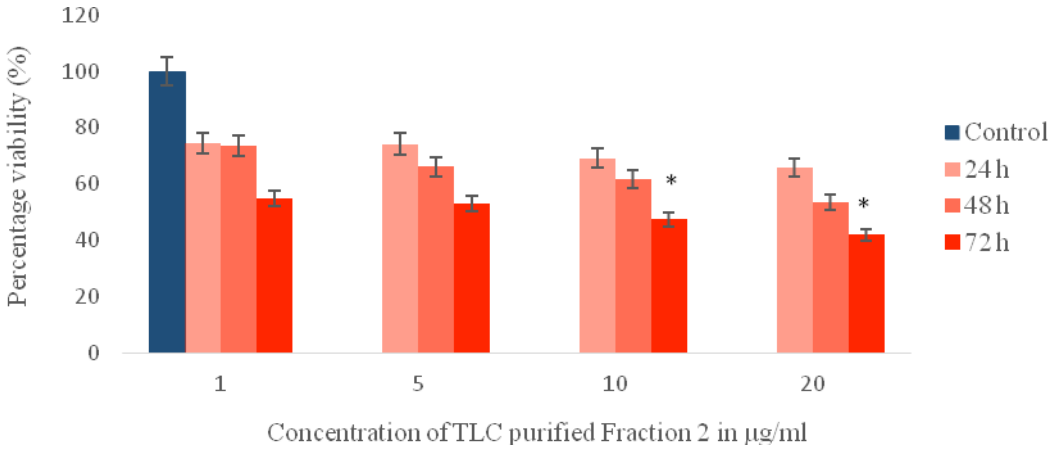

Figure 4 MTT assay of purified TLC fraction 2. Effect of the bioactive fraction 2 on HeLa cell viability when treated for 24, 48, and 72 h.(*$p<0.05$)

Further to determine the cell viability, trypan blue cell counting was performed which demonstrated a greater cytotoxicity on viable cells. Percentage cell viability was 14.28% after treating HeLa cells with 20µg/ml of extract for 24h.There was 100% cell viability observed in the control untreated cells (**Table 1**).

Table 1 Trypan blue cell counting

Sample	Live Cells	Dead Cells	Percentage Cell Viability (%)
Control	18	0	100
Test	3	18	14.28

DNA fragmentation is considered one of the hallmarks of apoptosis, occurring due to the significant biochemical changes [18]. Appearance of fragmented DNA of the extract treated cells on agarose gel as compared to the distinct band of the control cell is a clear evidence for the induction of apoptosis. DNA previously isolated from treated cells with 20µg/ml of bioactive fraction for 24h were visualized under UV.The gel showed a smear of DNA compared to the intact DNA of the control untreated cells (**Figure 5**).

LDH assay was performed to measure the Lactate dehydrogenase released into the media from damaged cells, which is a biomarker for cellular cytotoxicity. LDH activity was 1.7 times more in the supernatant of extract treated cells compared to the control supernatant (**Figure 6**). The activity of LDH present in the supernatant of the extract treated cells was higher than the control sample, demonstrating the cytotoxicity effect of the extract on HeLa cells.

Caspases have served as the primary mediators of apoptosis [19]. The caspase activity measured showed gradual increase in the activity of caspases form time t=0 to t=60 in extract treated cells in comparison to control cells. Treating with 20µg/ml of the bioactive fraction on HeLa cells showed a 1.4 fold increase in the final caspase activity as compared to the initial caspase activity. In the treated HeLa cells there was 44.51% more caspase activity than untreated cells after 60minutes, inferring the occurrence of apoptosis in extract treated cells (**Table 2**).

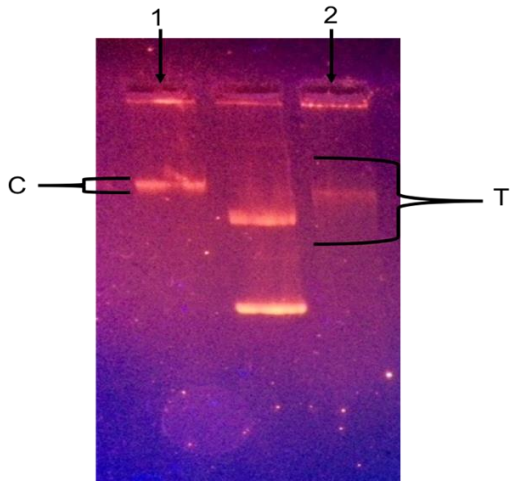

Figure 5 DNA fragmentation analysis of HeLa cells treated with the bioactive fraction 2. 1: Lane 1-DNA from control HeLa cells. 2: Lane 2- DNA from HeLa cells treated with 20 µg/mL of Fraction 2.C: Intact DNA.T: Smear of DNA indicating fragmentation

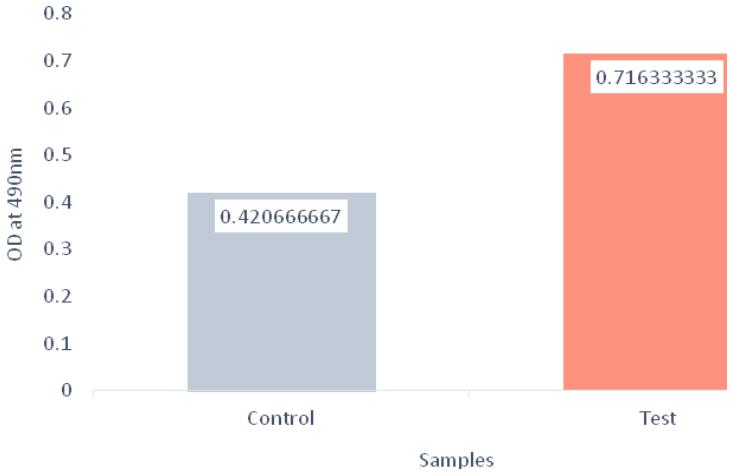

Figure 6 LDH assay of bioactive fraction 2. Effect of the bioactive fraction 2 on HeLa cell viability when treated for 24h

Table 2 Percentage activity of caspase-3 enzyme in HeLa cells treated with bioactive fraction 2 as compared to the controls

Sample	Optical Density at 500nm	Percentage (%)
Control	0.022	13.04
Treated	0.08	57.55
Blank	0	0

Conclusion

From the findings of the current study we can conclude that the TLC purified bioactive fraction from the *Penicillium* sp. has exhibited a promising cytotoxic and antiproliferative activity against Human cervical cancer cell line HeLa. From the various cytotoxicity assays conducted, it is evident that the growth and proliferation of HeLa cells is inhibited when treated with 20µg/ml of the bioactive fraction. This cytotoxic and antiproliferative effects demonstrated by the bioactive fraction could be due to the combined effect of induction of apoptosis mediated by caspases and direct cytotoxicity as per our current results. These results offer a platform for future research on to completely characterize this compound and to further conduct *in vivo* studies

Acknowledgements
The authors would like to thank the management of Jain Group of Institutions for the infrastructural facilities provided to carry out the work.

References

[1] G. M. Cragg, D. J. Newman, J. Ethnopharmacol., 2005, 100(1-2), 72-79.
[2] A. Klabatsa, S. Chicklore, S. F. Bamington. Goh, L. L. Lazdunski, G. J. Cook, Eur. J. Nuc. Med. Mol. Imaging., 2014, 41, 276-282.
[3] J. Maher, R. J. Brentjens, G. Gunset, I. Riviere, M. Sadelain, Nat. Biotechnol., 2002, 20, 70-75.
[4] A. J. Merina, D. Kesavan, N. Sulochana, Biotech. Rev. Lett., 2013, 1, 1-7.
[5] B. M. Selvi, S. Padmavathy, J. Brijitta, G. S. Kailiaraj, Int. J Biol. & Pharm. Sci., 2015, 2, 25-29.
[6] R. Deepa, S. Selvakumar, Chem. Sci. Rev. Lett., 2015 4(13), 148-152.
[7] S. Chanda, K. Nagani, J. Pharmacog. Phytochem., 2013, 2, 140-152.
[8] R. H. Cichewitz, S. A. Kouzi, Med. Res. Rev., 2004, 24(1), 90-114.
[9] T. Amagata, C. Rath, J. F. Rigot, N. Tarloy, K. Tenney, F. A. Valeriote, P. Crews, J Med Chem., 2003, 46(20), 4342-4350.
[10] A. L. Demain, A. Fang, Adv. Biochem. Eng. Biotechnol., 2000, 69, 1-39.
[11] A. Fleming, Bull World Health Organ., 2001, 79(8), 780-790.
[12] A. Debbab, H. A. Amal, H. L. Wen, P. Proksch, Microb. Biotechnol., 2010, 3(5), 544–563.
[13] A. M. Gamal-Eldeen, A. Ahmed-Lateff, T. Okino, Environ. Toxicol. Pharmacol., 2009, 28(3), 317-322.
[14] A. L. Demain, S. Sanchez, J. Antibiot., 2009, 62, 5- 16.
[15] V. K. Joshi, D. Attri, A. Bala, S. Bhushan, Ind. J. Biotech., 2003, 2, 362–369.
[16] T. Mosmann, J. Immunol. Methods., 1983, 65(1-2), 55-63.
[17] W. Strober, Current Protocols in Immunology, Trypan Blue Exclusion Test of Cell Viability, 2001, 21: 3B:A.3B.1–A.3B.2.
[18] X. Huang, H. D. Halicka, F. Traganos, T. Tanaka, A. Kurose, Z. Daezynkiewicz, Cell Prolif., 2005, 38(4), 223-243.
[19] D. W. Chang, Z. Xing, Y. Pan, A. Algeciras-Schimnich, B. C. Barnhart, S. Yaish-Ohad, M. E. Peter, X. Yang, EMBO J., 2002, 21(14), 3704–3714.

Publication History

Received	13th	Apr	2016
Accepted	05th	May	2016
Online	30th	Jun	2016