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**ANTI-PROLIFERATIVE EFFECTS OF *STREPTOCOCCUS* SP. JB24  
AND *CLADOSPORIUM* SP. JF9 METABOLITES ON HELA AND  
HEPG2 CELLS**

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**ABSTRACT**

This study was aimed to evaluate the potential cytotoxic activity of methanolic extracts of fungal and bacterial compounds against HeLa cervical cancer cell line and HepG2 liver cancer cell line. The cytotoxic activity of the extract was evaluated on HeLa and HepG2 cell lines using MTT assay method. Based on the morphological characters and chemical tests, the isolated microorganisms were found to be *Cladosporium* species (fungus) and *Streptococcus* species (bacteria). Thin layer chromatography (TLC) was performed to purify the compound present in the extract. Different concentrations (1, 10, 25, 50µg/mL) of the crude and TLC purified extracts were treated onto HeLa and HepG2 cancer cells which resulted in significantly inhibiting the proliferation of the cancer cells in a dose dependent manner. The cell number in the treated wells were exponentially lower when trypan blue assay was performed. These results suggest that both the isolated microorganisms contain secondary metabolites that possess anticancer property and hence responsible for the inhibition of the proliferating cancer cells.

**Key words:** *Cladosporium* sp., anti-proliferative, cytotoxic, metabolites, *Streptococcus* sp.

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## INTRODUCTION

Cancer is one of the life threatening diseases, characterized by uncontrolled proliferation of cells. Cancer harms the body when damaged cells divide uncontrollably to form lumps or mass of tissue called tumors, except in case of leukemia where cancer prohibits normal blood function by abnormal cell division in the blood stream [1]. Programmed cell death is called apoptosis, and when this process breaks down, cancer begins to form. Unlike regular cells, cancer cells do not undergo programmed death and instead continue to grow and divide. This leads to a mass of abnormal cells that grows out of control [2, 3 and 4]. The problem in managing this disease is not only due to its expensive cost of therapy but also the side effect following the use of nonselective chemotherapeutic agent [5]. Most of the drugs used in maintaining human health are extracted from natural sources such as plants and microbes [6]. The plants contain secondary metabolites that not only have anticancer property but also antimicrobial, antifungal, anti-inflammatory etc. [7]. But many such plants depend on environmental/climatic conditions for their growth. Microorganisms produce natural compounds that possess medicinal properties [8]. As microorganisms do not depend on climatic conditions for growth,

have the capability to produce secondary metabolites of therapeutic significance, cheap and easy to culture, the current study was focused on the use of microorganisms for the extraction of secondary metabolites having anticancer property.

## MATERIALS AND METHODS

### Isolation and identification

Soil and cow dung were collected and serially diluted with distilled water, spread onto Czapek dox media and Nutrient agar media for the growth of fungal and bacterial colonies respectively. Czapek dox agar plates were kept at room temperature for 4-5 days and Nutrient agar plates at 37 °C for 24hrs. Pure cultures of all the isolates were then maintained. Fungal identification was carried out based on the morphology of the fungal culture, the mechanism of spore production and the characteristics of the spores. Identification of bacteria was performed based on their morphology and biochemical characteristics according to the Bergy's manual of systematic bacteriology [9].

### Pigment extraction and partial purification

Fungal strains were inoculated aseptically in Czapek dox broth incubated at room temperature (25 °C -28 °C) for 7days and Bacterial strains were inoculated

aseptically in nutrient agar media and the plates were incubated at 37°C for 48h.

Fungal biomass was filtered and allowed to dry. Dried fungal biomass as well as bacterial biomass were macerated separately with methanol and the supernatant was collected by centrifugation. Supernatant was filtered using Whatman filter paper, solvent was evaporated at 60 °C, dried extract was dissolved in Dimethyl Sulphoxide (DMSO) and was used for further assays [10]. Thin layer chromatography (TLC) is a chromatography technique used to separate compounds present in the crude pigment extract. Pre coated Silica TLC sheets (60 F 254 Merck) were used to fractionate the pigment. Number of solvents like dichloromethane, chloroform, hexane, ethyl acetate, acetonitrile, acetone, butanol in different proportions tested as solvent system. After activating the TLC sheet at 110 °C for 15mins the extracts were spotted half centimeter above the bottom edge and allowed to run till the solvent system reaches half centimeter below the top edge. The distance moved by each fraction from origin was measured and corresponding Rf values were calculated. The separated fractions were detected under UV light. Marked each band on TLC sheet and scraped the band extracted with methanol [11].

#### Screening for Anticancer activities

The effect caused by crude extract and TLC purified fractions on the growth and proliferation of Human cervical cancer cell line (HeLa) and Human liver cancer cell line (HepG2) was tested by 3-(4,5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide) (MTT) cell viability assay, lactate dehydrogenase activity assay, DNA fragmentation assay and trypan blue cell counting assay. MTT assay was performed with HeLa and HepG2 cell lines. Cells were seeded onto 96 well flat bottom micrometer plates and incubate in a CO<sub>2</sub> incubator at 37°C with 5% CO<sub>2</sub> and 95% air overnight for cell adhesion. Different concentrations of crude and TLC partial purified pigments added in quadruplicates and incubated for 24, 48 and 72 hours. Following incubation 100ul of MTT solution was added. The culture was then incubated for 3hours in dark. After incubation period the supernatant was aspirated and 100ul of DMSO was added to dissolve the formazan. The absorbance was recorded at 540 nm with the help of ELISA plate reader [12]. The percentage of viability was calculated as per the standard methodology.

#### Trypan blue assay

The Trypan Blue assay was performed to determine the number of viable cells present in a cell suspension upon treating with TLC fractions. HepG2 cells were treated with different concentrations of the TLC

fractions for 24h [13]. At the end of the treatment period, the cells were trypsinized, were treated with 0.4% of trypan blue in phosphate buffer saline and counted with the aid of a haemocytometer [13]. The cell viability was calculated by the following formula:

$$\% \text{Viability} = \frac{\text{Number of transparent cells}}{\text{Total number of cells}} \times 100$$

### Lactate Dehydrogenase Activity (LDH) assay

Due to cytotoxicity Lactate dehydrogenase (LDH) enzyme released into the culture medium following loss of membrane integrity from either apoptosis or necrosis. Cytotoxicity of pigment analyzed by measuring the activity of LDH. Cancer cells treated with different TLC fractions for 24 hours were used to analyzed for LDH activity as per the instructions provided in LDH kit (G Biosciences Ltd, USA). Absorbance was taken at 490nm using a micro-plate reader and percentage of cytotoxicity calculated [14].

### DNA fragmentation analysis

HepG2 cells were cultured ( $1 \times 10^6$  cells/ml) in Roux flasks for 24h and treated with different concentrations of the TLC fractions. After 24 hours treatment period, cells were harvested, lysed and genomic DNA was isolated. Obtained genomic DNA suspended in TE buffer (20 $\mu$ l). 10 $\mu$ l of gel loading dye was added to it and sample electrophoresed using 0.8% agarose gel contain-

ing Ethidium Bromide. DNA bands were observed under UV transilluminator and photographed [15].

### Chemical screening of microbial extracts and active fractions

The crude extract and the TLC purified extracts were screened for presence of proteins, carbohydrates, glycosides, alkaloids, saponin, flavonoids, steroids and acid [16].

### Statistical analysis

All experiments were repeated thrice and 9 replicates were kept for each concentration. The mean $\pm$ SD was calculated. The results were analyzed statistically using one-way ANOVA and Student's t-test (unpaired test) (Prism 6 software) with 0.05 significance level ( $p \leq 0.05$ ).

## RESULTS

### Screening the isolates for cytotoxicity by MTT assay

In the present study we isolated few filamentous fungi and bacteria from various environmental sources in Bangalore and extracted the secondary metabolites using methanol as the solvent. When we screened our isolates for cytotoxicity on HeLa cells and on HepG2 cells, we found one of the Fungal isolates F9 inhibited viability of HepG2 cell line to 77.1% at a 1 $\mu$ g/ml concentration and 73.38% viability at 100 $\mu$ g/ml concentration (**Figure 1 A**). One bacterial isolate JB 24 (**Figure 1 B**) showed high anticancer potentials and inhibited the percentage viability of HepG2 cell line to

56.46% at a 1µg/ml concentration and 67.88% viability at 100µg/ml concentration.

#### Identification of isolates

JF-9 isolate identified as fungi by Lactophenol cotton blue staining and observed morphology as *Cladosporium* sp. (Figures 2A and 2B). JB-24 bacterial isolate found to be gram positive cocci in microscopic observation (Figures 2C and 2D) and belonging to *Streptococcus* sp. based on following biochemical observations like positive for Catalase test, negative for Mannitol Fermentation test, positive for Fructose Fermentation test and positive for 6.5% NaCl Salt tolerance test (Figures 3A, 3B and 3C).

#### Anti-proliferative activity of the microbial isolates

From this study Metabolites were extracted from JF9 and JB24 isolates using methanol. These extracts have shown significant cytotoxic and apoptotic potentials on cultured HeLa cells and HepG2 Cells. The green colored metabolite extracted from JF9-*Cladosporium* species inhibited the proliferation in a dose-dependent manner (from 93.88% to 77.10% and 72.03%) for HeLa cells and from 79.21%, 79.21% and 61.54% for HepG2 as the concentration of the extract increased from 1 to 10 and 50µg/mL respectively. Bacterial isolate, JB24-*Streptococcus* species also has shown

significant cytotoxicity to HeLa cells and HepG2 cells in a dose-dependent manner. At 1µg/mL, the percentage viability of HeLa cells was 75%, at 10 µg/mL it was 88.11% and at 25µg/mL concentration the percentage viability of HeLa cells was 75.87%. For HepG2, at 1µg/mL the percentage viability of the cells was 75.61%, at 10 µg/mL it was 66.63% and at 25µg/mL concentration the percentage viability was 72.11% (Figures 4A and 4B).

#### Thin Layer Chromatographic (TLC) fractionation of bioactive compounds

The methanol extract of *Cladosporium* sp. and *Streptococcus* sp. were subjected to partial purification by Thin Layer Chromatography (TLC) using dichloromethane:chloroform (80:20) as the solvent that resulted in 4 major fractions from both isolates (Figures 5A and 5B).

#### Cytotoxicity of TLC separated fractions

The four TLC separated fractions were again tested for cytotoxicity on HeLa and HepG2 cell lines. From *Cladosporium* sp. F4 and from *Streptococcus* sp. B4 fractions have demonstrated significant inhibition of cancer cell viability. Fraction F4 has inhibited the viability of HeLa cells to 74% at 1µg/mL, 66% at 10µg/mL and 67% at 25µg/mL concentrations. For HepG2 cells, at 1µg/mL the percentage viability of the cells was 85%, at 10 µg/mL it was 70% and at 25µg/mL concentration the percentage

viability was only 49%. The  $IC_{50}$  value was  $<10\mu\text{g/ml}$  on HepG2 cells (**Figures 6A and 6B**). Fraction B4 inhibited the viability of HeLa cells to 75% at  $1\mu\text{g/mL}$ , 71% at  $10\mu\text{g/mL}$  and 59% at  $25\mu\text{g/mL}$  concentrations. For HepG2 cells, at  $1\mu\text{g/mL}$  the percentage viability of the cells was 56%, at  $10\mu\text{g/mL}$  it was 55% and at  $25\mu\text{g/mL}$  concentration the percentage viability was 42%. The  $IC_{50}$  value for the B4 fraction was found to be  $2.252\mu\text{g/ml}$  on HepG2 cell line (**Figures 7A and 7B**). From these results it can be inferred that the isolates of *Streptococcus* sp. and *Cladosporium* sp in the current study have promising anticancer potential and can be explored further for characterizing the bioactive compound.

Trypan Blue dye exclusion method performed with  $25\mu\text{g/ml}$  concentration of B4 and F4 fractions on HeLa and HepG2 cell lines. F4 fraction reduced the cell concentration exponentially from  $8 \times 10^4$  cells/ml to  $7.25 \times 10^4$  cells/ml and B4 fraction reduced the cell concentration to  $6.25 \times 10^4$  cells/ml.

#### **LDH activity**

Lactate Dehydrogenase activity assay performed by adding  $25\mu\text{g/ml}$  of B4 and F4 fractions to the HepG2 cells. The OD values for the samples B4 and F4 were less

when compared to the Blank OD values (**Table.1**). Based on these results it can be reported that the extracts B4 and F4 do not exhibit any direct cytotoxic effects and the decrease in HepG2 cells viability found in our study could be due to the inhibition of proliferation of cancer cells.

#### **DNA fragmentation**

If the cells are undergoing apoptosis, then the DNA of the cells will appear fragmented. When the DNA extracted from HepG2 cells treated with  $25\mu\text{g/ml}$  concentration of B4 and F4 fractions were subjected to agarose gel electrophoresis, only B4 fraction showed DNA fragmentation and F4 fraction has not shown any significant DNA fragmentation. These results indicated that the fraction B4 from *Streptococcus* sp. was able to induce apoptosis of the treated cancer cells and hence has more potent anti-cancer activity than the fungal fraction F4 (**Figure 8**).

#### **Chemical screening of crude and TLC purified extracts**

Chemical composition screening indicates presence of alkaloid and a glycoside in F4 fraction. B4 fraction from the bacterial isolate, *Streptococcus* sp., showed the presence of an alkaloid compound (**Table 2**).

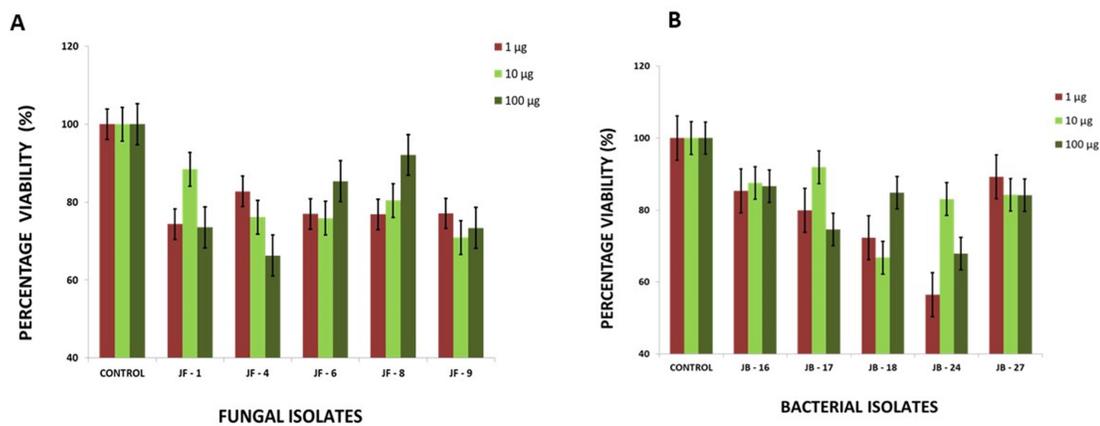


Figure 1 (A): Percentage viability of HepG2 cells treated with Fungal extracts. (B): Percentage viability of HepG2 cell line treated with bacterial extracts

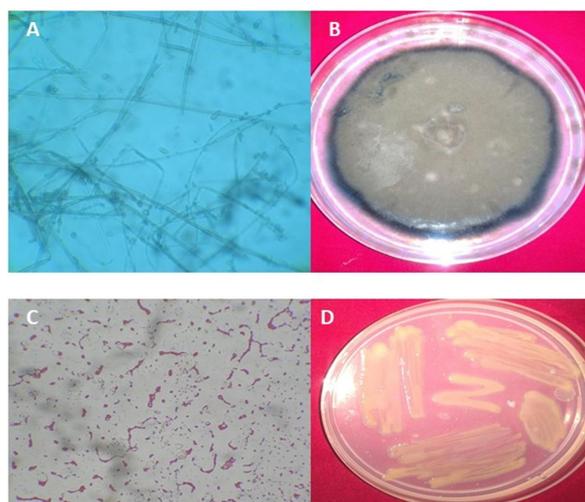


Figure 2(A): Microscopic observation of the Fungus (B): Fungal pure culture plate (C): Microscopic observation (D): Pure culture of bacterial isolate

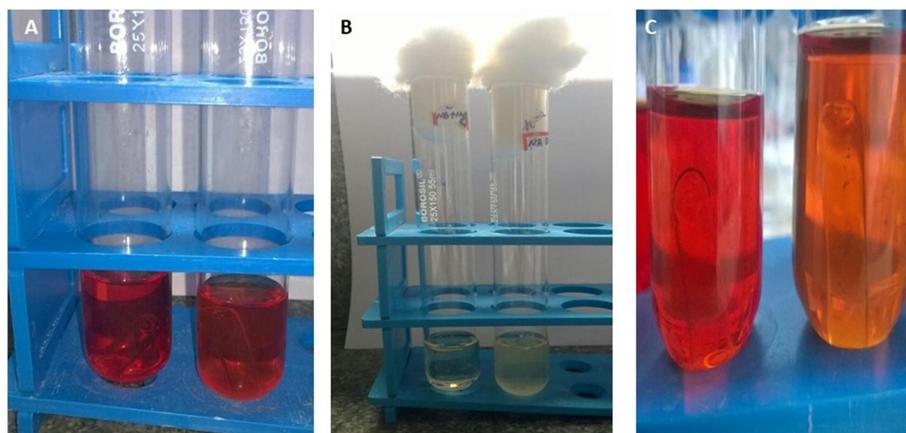


Figure 3(A): Mannitol Fermentation Test (B): Salt Tolerance Test. (C): Fructose Fermentation Test.

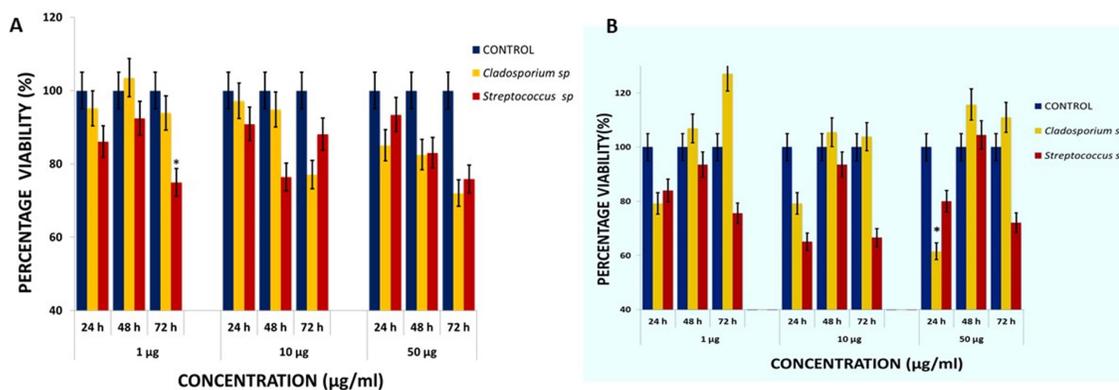


Figure 4: Percentage viability of (A) HeLa cell line treated with *Cladosporium* and *Streptococcus sp.* methanolic extracts for 24, 48 and 72 h. (B): HepG2 cell line treated with *Cladosporium* and *Streptococcus sp.* methanolic extracts for 24, 48 and 72 h.

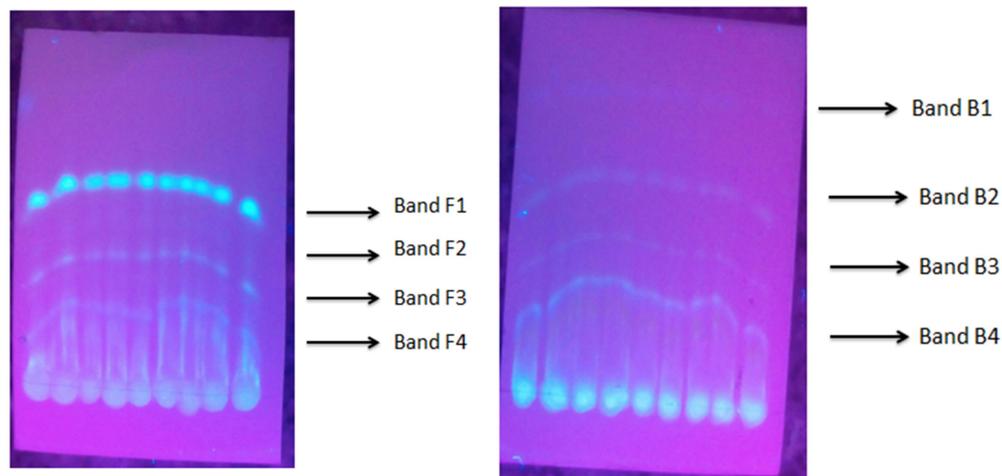


Figure 5 (A): TLC of *Cladosporium* sp. extract as observed under UV and (B)TLC of *Streptococcus* sp. extract as observed under UV.

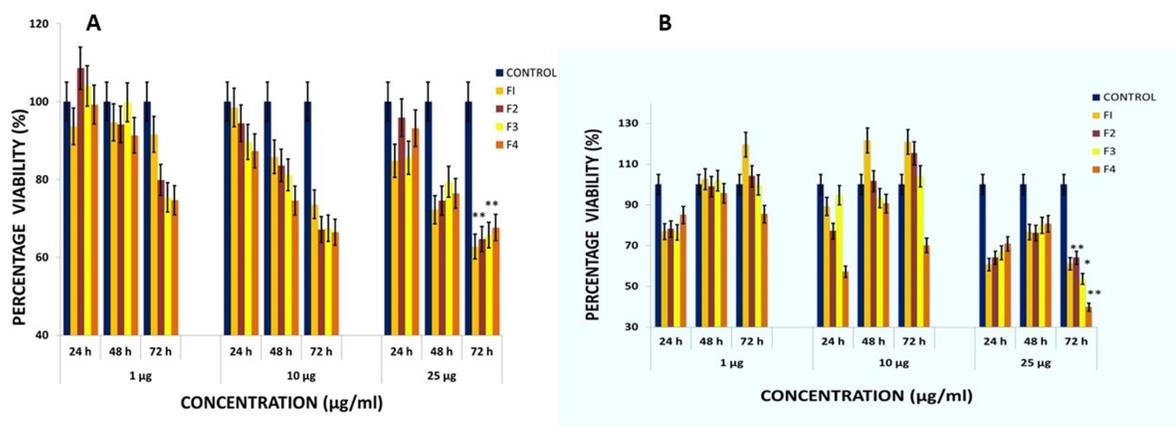


Figure 6 (A): Effect of TLC purified fungal fractions (F1, F2, F3, F4) on HeLa cell line for 24, 48, 72h. (B): Effect of TLC purified fungal fractions (F1, F2, F3, F4) on HepG2 cell line for 24, 48, 72h.

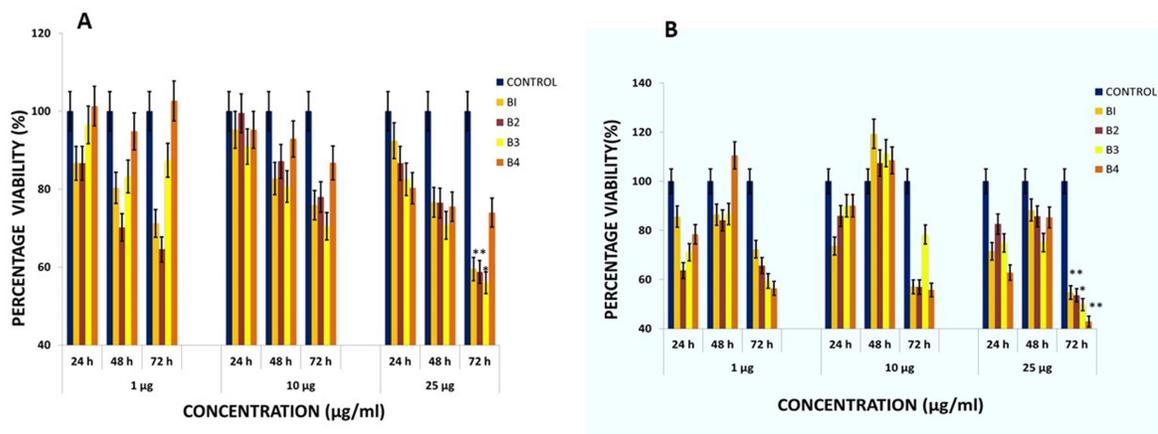


Figure 7 (A): Percentage viability of HeLa cells treated with TLC fractions of bacteria after 24, 48, 72 h (B): Percentage viability of HepG2 cell line treated with TLC fractions of bacteria after 24, 48, 72 h

Table 1: OD values of samples for LDH assay at 490 nm.

Sample	Control	Blank	B4	F4
Absorbance at 490 nm	1.503	0.588	0.398	0.383
	1.535	0.585	0.313	0.350

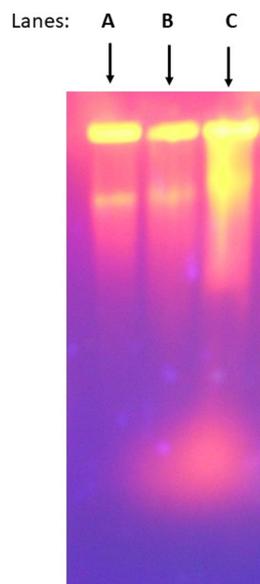


Figure 8: Analysis of DNA on 0.8% Agarose gel electrophoresis. Lanes: A - DNA from Control HepG2, B-DNA from

fungal fraction (F4) treated HepG2 & C- DNA from bacterial fraction (B4) treated HepG2 cells

Table 2: Chemical screening of crude and TLC purified extracts

TESTS	FUNGAL (F4)		BACTERIAL (B4)	
	Crude	Fraction	Crude	Fraction
Saponin	-	-	-	-
Glycoside	+	+	-	-
Steroid	+	-	-	-
Ninhydrin	+	-	+	-
Carbohydrates	-	-	-	-
Flavonoid	-	-	-	-
Alkaloid	+	+	+	+
Acid	-	-	-	-

## DISCUSSION

Recent investigations have been intensified by the potentialities of endophytic fungal strains in the production of pigments, bioactive metabolites, immune-suppressants, anticancer compounds and biocontrol agents [17]. Many fungal secondary metabolites with various chemical structures and their wide ranging biological activities were reported, which reflects the high synthetic capability of fungi [18]. About 1500 fungal metabolites have been reported to show anti-tumor and antibiotic activity and some have been approved as drugs. It has been estimated that there may be 1.5 million fungal species, while only about 100,000 species are presently known [19]. Very few reports are available on the anticancer compounds from *Streptococcus* species where they assessed the anticancer activities of the peptidoglycans or membrane components of the cell walls of various LAB strains, including *Lactobacillus*, *Lactococcus*, *Streptococcus*. Soluble polysaccharide fraction of the HK cells of *L. aci-*

*dophilus* 606 was found to inhibit different cancer cell like HeLa, MCF7, U-87, HepG2, U2OS and PANC-1 proliferation by 20%. Moreover, these polysaccharides proved to be much less cytotoxic to normal cells than the whole HK cells of the same strain [20].

Some endophytic fungal crude extracts exhibited cytotoxicity against the human myeloid leukemia cells and murine leukemia cells with IC<sub>50</sub> values ranging from 0.01 µg/ml to 100 µg/ml concentration. But they have not subjected these crude extracts for further purification or characterization studies [21]. Another study reported that taxol from the endophytic fungus *Lasiodiplodia theobromae* isolated from the medicinal plant *Morinda citrifolia* was cytotoxic to MCF-7 cell line with an IC<sub>50</sub> value of 300 µg/ml [22]. In a previous study, ethyl acetate extracts from eighty-four representative endophytic fungal species were checked for anticancer activities by MTT assay against A375 (human malignant melanoma), SW620 (human colorectal adeno-

carcinoma), Kato III (human gastric carcinoma), HepG2 (human liver hepatoblastoma) and Jurkat (human acute T cell leukemia). Twenty six (30.95%) isolates had cytotoxic activity against some cancer cell lines. Two isolates (*Pestalotiopsis* sp. 1 and *Cladosporium* sp. 1) had broad-spectrum inhibition against all cancer cell lines. Seven isolates had specific anticancer activities against Jurkat cells, with cell viability below 40%. Crude extract of *Cladosporium* sp. 1 had the highest anti-microbial and anticancer potential [23]. Hence, the extracts from our isolated fungi and bacteria seem to be having more potent cytotoxicity than these earlier reports.

### CONCLUSION

We can conclude that the secondary metabolites from our isolates of fungi (*Cladosporium* sp.) and bacteria, *Streptococcus* sp. exhibited significant anti-proliferative effects against cultured HepG2 and HeLa cells and can serve as potential sources for the production of bioactive components having promising anticancer activities. Further studies are warranted to completely characterize these isolates and their bioactive compounds towards anticancer application studies.

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### CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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