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ORIGINAL ARTICLE

In vitro Antiproliferative Activity of Extracellular Pigment from *Streptomyces* sp. BS19 against T-Cell Leukemia and B Cell Lymphoblast Cell Lines

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ABSTRACT

The present study evaluated the In vitro antiproliferative activity of extracellular pigment from Streptomyces sp. BS19 against liquid cancer cell lines. Extracellular pigment from bamboo rhizosphere soil Streptomyces sp BS19 was obtained by agar surface fermentation using YEME agar. In vitro antiproliferative activity of pigment was tested by resazurin assay in T-cell leukemia (Jurkat) and B cell lymphoblast (Z138) cell lines as well as in normal human peripheral blood mononuclear cells (PBMC). The effect of crude pigment on apoptosis, cell cycle and some key signaling proteins was also evaluated. The results showed that crude pigment exhibited significant anti-proliferative activity in lymphoma (Z138) and leukemia (Jurkat) cell lines with an IC50 of 0.152 ng/ml and 0.258 ng/mL respectively. The crude pigment showed several-fold less activity (IC50 of 163 ng/mL) in normal human PBMCs. The crude pigment showed a significant increase in sub-G0 cells when compared to control at 48 hours. Significant downregulation of c-Myc and phospho ERK 1/2 and increased cleaved PARP was shown by western blot in crude pigment treated samples. Cell cycle analysis shows the accumulation of cells in the sub-G0 phase in pigment treated cells indicating apoptotic mediated cell death. The apoptosis mediated cell death is also supported by an increase in cleaved PARP. Thus, the present work highlights extracellular pigment from Streptomyces sp. in bamboo rhizosphere soil as a promising anticancer agent for leukemia and lymphoma.

Keywords: *Streptomyces sp, pigment, Leukemia, Lymphoma, antiproliferative, apoptosis.*

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INTRODUCTION

Cancer is a disorder characterized by uncontrolled cell division. The molecular mechanisms which transform the normal cell to divide in an uncontrolled manner are contributed by environmental factors, genetic abnormalities, and life-style disorders. The incidence of cancer has been on the rise and the recent 2020 WHO report estimates that there will be about 20 million cases worldwide in the next 17 years if control measures are not taken right away (WHO Report, 2020). Cancer is a complex disease that is managed by a plethora of drugs with a different mechanism of action to deal with the burden of the disease. Cancers occuring in blood cells are called hematological cancers which are heterogenous and accounts for 6% of all the cancers as per GLOBOCAN 2018 report. The types of hematological cancers are leukemia, lymphoma and multiple myeloma. Though there are several drugs to treat cancer including hematological cancers, the burden of cancer is rising and challenged by resistance to standard of care drugs [1-2] and by exhibiting cardiotoxicity [3]. Hence it is becoming inevitable to continue exploring for

additional agents having anticancer activity. In the field of cancer drugs, there were about 247 drugs approved from the year 1980 to 2019. Among the 247 approved drugs, 41% of them are natural products, derivatives, or inspired by natural pharmacophore [4].

Members of the Phylum actinobacteria are the tremendous source for bioactive natural product discovery as well as for other biotechnological applications [5]. Notably, members of the genus *Streptomyces* are well recognized as the source for antibiotics [6-7]. Among the anticancer natural products, metabolites from the *Streptomyces* sp have been significant contributors [8]. Pigmented secondary metabolites are explored with more interest because they are produced in response to challenging environments, and they possess interesting bioactivities. *Streptomyces* sp are known to produce pigmented secondary metabolites which have shown excellent antimicrobial, immunosuppressive and anticancer activities [9]. Some of the earliest pigments discovered from *Streptomyces* sp are peptides and anthracyclines which are developed into successful anticancer drugs such as Actinomycin D, Bleomycin, and Doxorubicin [10]. Progidiosins, linear tri-pyrrole pigments discovered in *Streptomyces* sp possess anticancer properties and are evaluated in clinical trials [11]. Nearly 45% of the bioactive metabolites are obtained from the *Streptomyces* sp which promises to explore this species progressively [12]. There are recent reports of extracellular pigments from *Streptomyces* sp having anticancer [13] and antimicrobial activity [14-15]. In this study, the observed anti-proliferative activity exhibited by an extracellular pigment from *Streptomyces* sp BS19 isolated from bamboo rhizosphere soil against lymphoma (Z138) and leukemia (Jurkat) cell lines is described.

MATERIALS AND METHODS

Description of *Streptomyces* sp. BS19

The *Streptomyces* sp. BS19 which produce extracellular yellow-orange pigment was isolated from the bamboo rhizosphere soil collected from Megamalai forest region (Lat. 9.64612˚ N; Long. 77.40134˚ E), Tamil Nadu, India using starch casein nitrate agar. Viability of the strain BS19 was maintained on ISP2 agar slants as well as in 30% glycerol broth at 4^{o}C and -20^{o}C, respectively.

Production and extraction of pigment from strain BS19

Spores of strain BS19 were inoculated into 20 ISP2 agar plates and incubated at 28^{0}C for 10 days. After incubation, the mycelial growth was removed using sterile spatula and discarded. Then the agar medium was cut into pieces and the extracellular pigment present in that was extracted using ethyl acetate at 1:2 ratio for 24 hours at room temperature. The solvent portion was concentrated using the rotary evaporator [15]. The dried crude extract of extracellular pigment was collected in a screw cap vial and used for antiproliferative studies.

Cell Culture

Acute T-cell leukemia (Jurkat) and Lymphoma of B-lymphoblast (Z138) cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Jurkat cell line was maintained in Roswell Park Memorial Institute Medium (RPMI). The culture media was supplemented with 10% heat-inactivated Fetal Bovine Serum (GIBCO, USA), 100 units/mL Penicillin and 100µg/mL Streptomycin (GIBCO, USA). Z138 cell line was maintained in Iscove's Modified Dulbecco's Media (IMDM) supplemented with 10% heat-inactivated horse serum (GIBCO, USA), 100 units/mL Penicillin and 100µg/mL Streptomycin. The cell lines were maintained in 5% CO_2, and at 37^{0}C temperature. The cell lines were tested for any contamination of mycoplasma using the mycoplasma detection kit (Lonza, Alpharetta, GA, USA) following the manufacturer's protocol.

Isolation of normal peripheral blood mononuclear cells

A blood sample was collected from a healthy volunteer according to the institutional ethics committee. Normal peripheral blood mononuclear cells (PBMCs) were isolated from the blood of a healthy donor using Histopaque-1077 (Sigma-Aldrich) using the supplier's protocol. The isolated PBMCs were cultured in RPMI media supplemented with 10% heat-inactivated Fetal Bovine Serum (GIBCO, USA), 100 units/mL Penicillin and 100µg/mL Streptomycin (GIBCO, USA).

Cell Proliferation assay

Effect of extracellular pigment on cell proliferation using resazurin assay

The antiproliferative activity of crude pigment was studied by adopting resauzurin assay [16, 17]. The cells, Z138 and Jurkat, were seeded at a density of 10,000 cells / well in 90µL volume in 96 well round bottom plate. The cells were treated with the crude pigment diluted in DMSO at 1000, 100, 10, 1, 0.1, 0.01, 0.001, 0.0001, 0.00001 ng/mL concentrations. In control wells, the cells were treated with 0.1% DMSO. Following pigment addition, the cells were incubated for 72 hours. To the cells with media, 50µL of resazurin dissolved in PBS at a concentration of 0.05 mg/mL was added. Resazurin is a dye when added to cells gets reduced by NADPH present in mitochondria and gets converted to a pink fluorescent product

resarufin. Thus, the conversion is directly proportional to the number of viable cells. The fluorescence is measured in a spectramax 190 microplate reader (Molecular devices LLC, USA) with an excitation wavelength of 535nm and an emission of 595nm. Cell viability was calculated as a percentage of fluorescence measured in the pigment treated wells relative to the DMSO control wells. IC_{50} values were determined as concentrations that reduced cell viability by 50% and the curve was plotted with Graphpad Prism 6.0 software (San Diego, USA). The experiment was performed in triplicates and the mean and standard deviation were calculated. Percent inhibition of Proliferation was calculated as follows.

Percent Inhibition of Proliferation = 100-(Relative fluorescence of treated/Relative fluorescence of control)*100.

Cytotoxicity assay in human PBMCs

Human PBMCs were seeded at a density of 1 X 10^5 cells per well in 96 well round bottom plate. The cells were treated with the crude pigment diluted in DMSO and added to the cells to achieve final concentrations of 1000, 100, 10, 1, 0.1, 0.01, 0.001, 0.0001, 0.00001ng/mL in cells. In control wells, the cells were treated with 0.1% DMSO. Following the addition of crude pigment, the cells were incubated for 72 hours. Post 72 hours the viability of the cells was assessed using resazurin assay as described in the cell proliferation assay section. Cell viability was calculated as a percentage of fluorescence measured in the pigment treated wells relative to the DMSO control wells. IC_{50} values were determined as concentrations that reduced cell viability by 50% and the curve was plotted with Graphpad Prism 6.0 (San Diego, USA). The experiment was performed in triplicates and the mean and standard deviation were calculated. Percent killing of cells was calculated as follows.

Percent Cytotoxicity = 100-(Relative fluorescence of treated/Relative fluorescence of control)*100.

Cell Cycle Analysis by FACS

Z138 cells were seeded at a density of 1 X 10^6 per/mL in a 24 well plate. The cells were treated with crude pigment for 48 hours. Post 48 hours, the cells were harvested by centrifuging at 1200rpm for 10 minutes. The cells were washed with 1X Phosphate buffered saline and fixed with 70% ice-cold ethanol for 30 minutes. The fixed cells were washed twice with 1X PBS. The cells were treated with 50µg/mL of propidium iodide (Sigma) and 100 µg/mL of RNAase at room temperature for 30 minutes. The stained cells were analyzed with FACS caliber flowcytometer (BECTON DICKINSON, USA).

Evaluation of extracellular pigment activity on C-Myc, phospho-ERK1/2, and PARP by Western blotting

Z138 cells were seeded in 6-well plates and treated with the crude pigment for 24 hours. Cells were harvested and lysed with cell lysis buffer having the composition of 50mM HEPES (pH 7.4), 150mM of sodium chloride, 5mM of EDTA, 1% of Triton X100 supplemented with protease and phosphatase inhibitor cocktails (Sigma, USA). Protein from the lysate was quantified with Pierce BCA Protein Assay Kit (Thermo Scientific, USA) following the supplier's protocol. 50µg of protein was subjected to a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane (GE Healthcare, USA). The membrane was blocked with Licor blocking buffer (LI-COR). After blocking, the membrane was probed with primary antibodies (PARP, c-Myc, phospho ERK 1/2, and beta-actin) for overnight at 4^0C. After overnight incubation, the binding of the primary antibodies was detected with IRDye 800CW Goat anti-Rabbit IgG (LICOR-926-32211) and 680 RD anti-mouse IgG (LICOR-926-68070) secondary antibodies. The blot was developed with the LI-COR Odyssey 9120 Digital Imaging System. The primary antibodies PARP, phospho ERK1/2, and c-Myc were obtained from Cell Signaling Technology with catalog numbers 9542, 4370, and 9402. The beta-actin antibody was from Santa Cruz Biotechnology, Inc with catalog number Sc-69879.

RESULTS

Anti-proliferative activity of extracellular pigment

Extracellular pigment exhibited a particularly good dose-dependent inhibition of cell proliferation with an IC50 of 0.152 and 0.258 ng/mL in Z138, and Jurkat cell lines respectively (Figure 1). Doxorubicin was used as controls in proliferation experiments. The extracellular pigment activity was further tested in normal PBMCs (healthy volunteers) where it showed lesser activity (163ng/mL) when compared to activity in cancerous cell lines, indicating that the extracellular pigment has good selectivity on inhibiting the proliferation of cancer cells over normal cells (Figure 2).

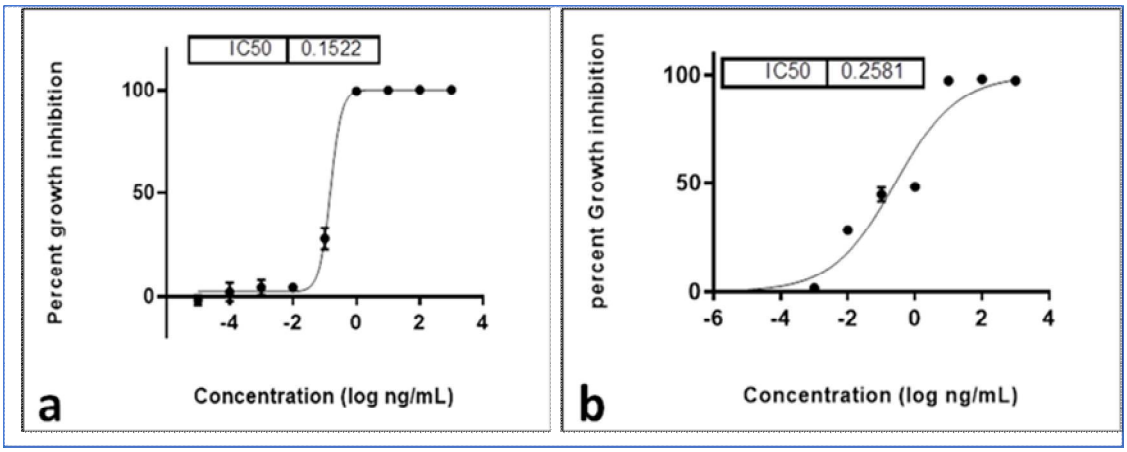


Figure 1. Effect of extracellular pigment on proliferation of (a) Z138 and (b) Jurkat cell lines.

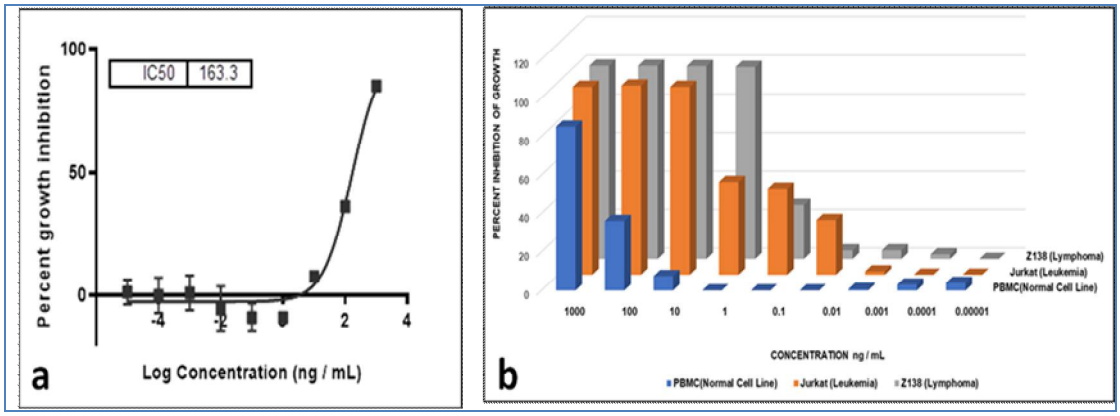
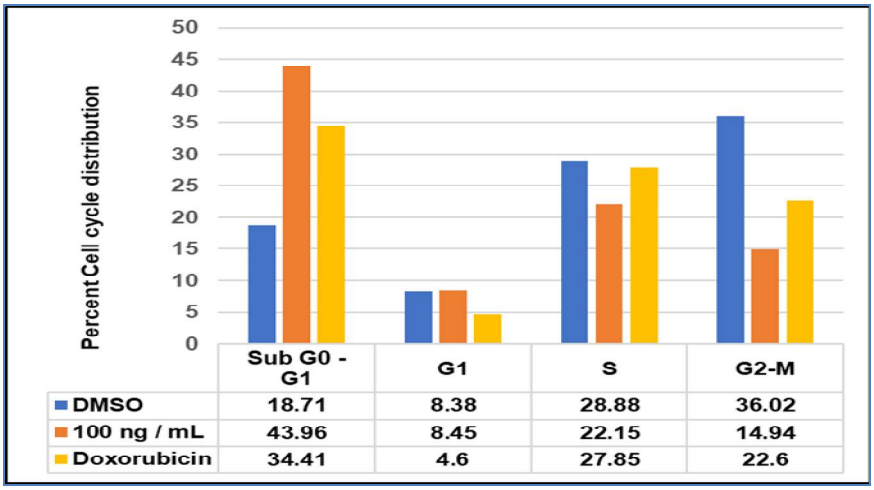


Figure 2 (a). Effect of extracellular pigment on PBMC.
(b) Comparison of Percent inhibition of proliferation of by extracellular pigment in cancer cell lines versus normal cells.

Effect of extracellular pigment on cell cycle

Z138 cell line was treated with extracellular pigment for 48 hours and the distribution of cells in different phases of the cell cycle was analyzed. Treatment with 100 ng/mL extract leads to a 2.3-fold increase in the sub-G0 population of cells when compared with DMSO control (Figure 3). Doxorubicin treatment also resulted in a significant increase in sub-G0 population cells as expected.



	Sub G0 - G1	G1	S	G2-M
DMSO	18.71	8.38	28.88	36.02
100 ng / mL	43.96	8.45	22.15	14.94
Doxorubicin	34.41	4.6	27.85	22.6

Figure 3. Effect of extracellular pigment on cell cycle in Z138 cell line.

Effect of extracellular pigment activity on signalling proteins C-Myc and phospho-ERK1/2

We further evaluated the potential downstream targets in Z138 cells treated with extracellular pigment. Signaling proteins such as c-Myc, pERK 1/2 were assessed. Upon treatment, the expression of c-Myc was significantly down-regulated in a dose-dependent manner with significant inhibition at 10ng/mL (around 50%) in the Z138 cell line (Figure 4). Moderate inhibition of pERK1/2 (48 %) observed in Z138 at 10ng/mL (Figure 4).

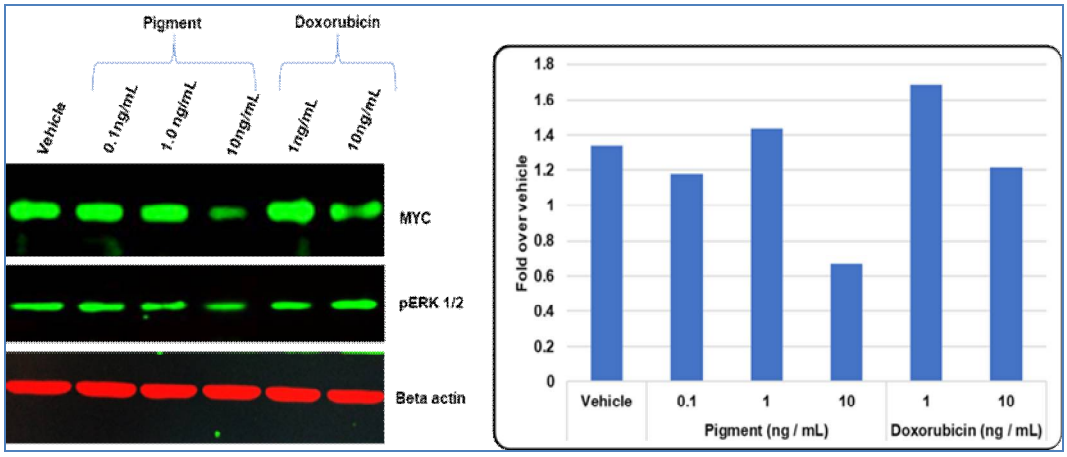


Figure 4. Effect of extracellular pigment on c-Myc and pERK 1 / 2 levels in Z138 cell line.

Effect of extracellular pigment on apoptotic proteins

The effect of extracellular pigment on apoptotic protein Poly (ADP-ribose) polymerase (PARP) was evaluated by western blot in Z138. A dose-dependent increase in cleaved PARP levels (0.9, 1.9 and 6.7-fold increase at 0.1, 1, and 10ng/mL over DMSO control) was observed upon treatment of the Z138 cell line with the pigment (Figure 5).

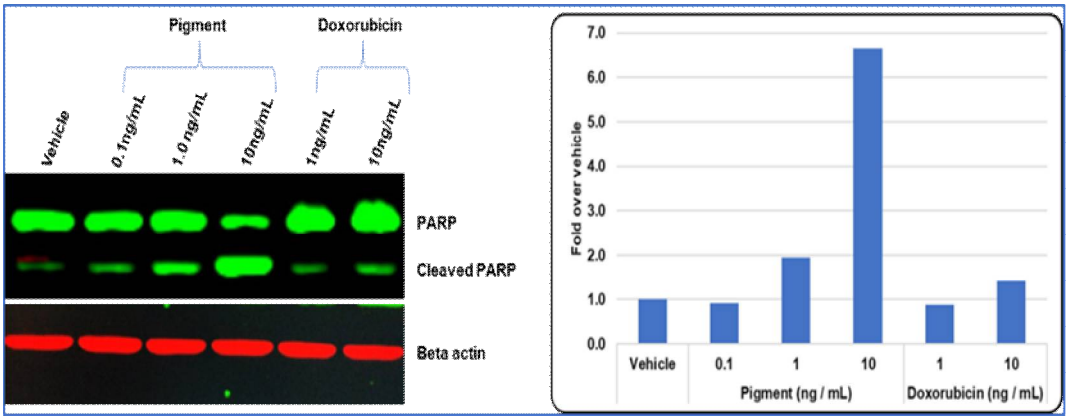


Figure 5. Effect of extracellular pigment on PARP levels in Z138 cell line.

DISCUSSION

Streptomyces sp are known to produce a wide range of colored pigments in response to their quorum, that aid the species to protect it from UV radiation, biofilm-producing microorganisms, and competing microflora. Historically, pigments with antibiotic activity exhibited good anticancer activity also [18-19]. Pigments have significantly contributed as anticancer drugs, such as doxorubicin [20] and the recent one being romidepsin [21-22]. The success in obtaining anticancer compounds from *Streptomyces* sp of terrestrial origin has kept the exploration ongoing. There are reports of pigmented secondary metabolites with anticancer property from *Streptomyces* sp of diverse origin such as humus from Western Ghats [23] coral reef environment [24], mangrove ecosystem [25] soil and rhizosphere soil [15, 26-27].

In this study, extracellular pigment isolated from *Streptomyces* sp of bamboo rhizosphere soil has shown particularly good anti-proliferative activity against leukemia and lymphoma cell lines.

According to the NCI guideline (NCI, USA), any natural product having an antiproliferative activity of less than 30ug/mL in the initial screening in cell lines is interesting to explore further. In this study, the

extracellular pigment isolated from *Streptomyces* sp of rhizosphere soil has exhibited nanomolar potencies in Z138 and Jurkat cell lines. Leukemia and lymphoma are among the top 15 in the list of cancer deaths in the world [28]. The extracellular pigment has shown more selectivity to cancer cells. The fold selectivity over normal human PBMCs is 1072 and 631 respectively in Z138 and Jurkat cell lines. *Streptomyces* sp isolated from the bamboo rhizosphere showing antimicrobial activity has been reported earlier [29-30]. Anticancer activity in solid tumors has been reported with *Streptomyces* sp isolated from the rhizosphere of mangrove [31], medicinal plants [32] and Antarctic hairgrass [33]. There are reports of extracellular pigments from rhizosphere soil active against hematological cell lines [34-36]. It is interesting to observe in our study the extracellular pigment derived from bamboo rhizosphere *Streptomyces* sp has exhibited more potency in hematological cell lines when compared with reported extracellular pigments obtained from mangrove and marine ecosystem. One of the hallmarks of cancer is resisting cell death [37]. Hence inducing programmed cell death is one of the preferred characteristics of anticancer drugs. The extracellular pigment was evaluated for its apoptotic activity upon treatment in the Z138 lymphoma cell line. There was a significant increase in the apoptotic population when treated with extracellular pigment. The hallmark among the apoptosis-induced cell death is the cleavage of Poly (ADP Ribose) Polymerase [38-39] by caspase which results in 85kDa and 25 kDA fragments. The 85 kDa fragment is involved in the programmed cell death process. The extracellular pigment has shown cleavage of PARP in a dose-dependent manner indicating that the cell death is mediated through apoptosis.

The extracellular pigment was also evaluated for its effect on MYC. MYC a proto-oncogene is one of the most dysregulated genes in more than 50% of cancers and accounts for transcription of 15% of genes from the genome [40]. MYC regulates transcription of genes involved in cell proliferation, apoptosis, and differentiation [41]. MYC is upregulated in hematological cancer and it is an interesting target in hematological cancer. Z138 cells when treated with extracellular pigment has shown downregulation of c-Myc levels in a dose-dependent manner. A significant inhibition is observed at 10 ng/mL.

Streptomyces sp have contributed plenty of anticancer agents which are approved as drugs and drug candidates in clinical trials [42, 43]. Secondary metabolites derived from *Streptomyces* sp from extreme environments are having unique characteristics that contribute to their anticancer property. The rhizosphere is a region where there is rich interaction between the roots and the microbial flora [44]. The bamboo rhizosphere is one such unique environment due to its high biomass and efficient carbon fixing property having *Streptomyces* sp as a dominant microflora would possess interesting secondary metabolites.

The extracellular pigment from *Streptomyces* sp has exhibited favorable characteristics of an anticancer agent. The pigment exhibits significant antiproliferative activity with selectivity over normal cells. It induces apoptosis and down-regulates c-Myc one of the predominantly dysregulated genes in cancers making it an ideal candidate to be explored for potential anticancer drug.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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