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EVALUATION OF THE *IN-VITRO* ANTICANCER ACTIVITY OF THE RED PIGMENT SR6 FROM *SERRATIA MARCESCENS* JGI 27

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ABSTRACT

Pigments and secondary metabolites from microbes have been extensively studied for their therapeutical potential. The anticancer activity of red pigment (SR6) from *Serratia marcescens* JGI 27 (isolated from soil of Bangalore) has been evaluated against HepG2 and Jurkat cell lines by various *in vitro* assays. The purification of crude extract was performed by thin layer chromatography (TLC) and we could isolate 9 different fractions which were tested for their cytotoxicity by MTT assay. Out of nine fractions, red fraction from *S. marcescens* JGI 27 (SR6) had exhibited maximum antiproliferative effect on HepG2 and Jurkat cells with an IC_{50} of 16.08 and 16.54 µg/ml respectively. Later, cytotoxicity was observed and confirmed through DNA fragmentation analysis, caspase 9 apoptotic assay and LDH analysis. The cytotoxic effect of SR6 were tested on normal cells (CHO and lymphocytes) and found to be least toxic. This study shows that SR6 has anticancer potential and it can further be explored for identification and characterisation studies.

Keywords: Cytotoxicity, HepG2 cells, Jurkat cell, CHO, SR6, *Serratia marcescens JGI 27*

INTRODUCTION

Cancer is one of the major health problems in developing countries and the identification of novel targets and the development of more specific chemotherapeutic agents are the most important goals of research in cancer therapy **[1]**. *S.marcescens*, a gram negative bacterium characterized by production of a red pigment prodigiosin, a tripyrrole compound **[2]**. Prodigiosin has been known

to be a natural compound showing diverse biological activities such as anti-bacterial, anti-malarial, anti- tumor, immunosuppressive and cytotoxic activities **[3, 1]**. The cytotoxic and antiproliferative activity of prodigiosin have been observed not only in cultured tumour cell lines but also in human primary cancer cells and interestingly it has no marked toxicity in non-malignant cell lines **[4]**. Most of the cancer therapies involve elimination of the cancer cell by apoptosis through activation of caspases, however, apoptosis may not be the only mode of cancer cell death by prodigiosin. Induction of cell death through caspase independent and other modes of cell death also have an important role in cytotoxic therapy **[5, 6]**. The antiproliferative effect of prodigiosin is p^{53}-independent that makes prodigiosin a new antineoplastic agent to study in cell culture cancer models **[7]**.

In the present study we tried to evaluate the potential cytotoxic effect of the red pigment (SR6) from an isolate of *S. marcescens* against HepG2 and Jurkat cell lines by various *in-vitro* assays and also to evaluate their effects on normal peripheral lymphocytes and CHO cells.

MATERIALS AND METHODS

Cell line and culture conditions

From NCCS (National Center for Cell Science), Pune, HepG2 (liver cancer), Jurkat (leukemia) and CHO (normal ovarian) cell lines were procured. MEM (Modified Eagle's medium, HiMedia Laboratories, Mumbai, India) and RPMI-1640 (HiMedia, India) were used for maintaining the cells, supplemented with 10% FBS (Fetal bovine serum, HiMedia), 100 µg/ml of streptomycin and 100 U/ml of penicillin. Cells were incubated at 37℃ in a humified incubator with 95% air and 5% CO_2.

Isolation and identification of bacteria

Bacteria were isolated from soil sample of Bangalore, India. The collected soil samples were serially diluted and plated to on nutrient agar plates. Colonies of bacteria showing red coloration were isolated to maintain pure cultures and stored at 4℃. Red pigmented bacteria were identified by biochemical characterisation based on Bergey's classification of determinative bacteriology.

Isolation of lymphocytes

From the healthy individuals, blood was collected following the ethical guidelines of research from ICMR. HiSep medium (HiMedia) and RPMI-1640 media were used for isolation and suspension of lymphocytes. The media was supplemented with 5mg/ml PHA (phytohemagglutinin) and 10% FBS. Cells were maintained in a 5% CO_2 humified incubator at 37℃.

Pigment extraction and purification

By following the standard protocols, pigment was extracted from the bacterial isolate using methanolic extraction method **[8]**. Crude extract was subjected to purification by thin layer chromatography (TLC) using the solvent system, acetone: ethyl acetate (1:1). The silica gel pre-coated TLC sheet (60 F 254, Merck) was activated at 110℃ for 30 min and the crude pigment extract was spotted at the bottom of the silica gel sheet. With the help of UV transilluminator (254 and 366nm), chromatograms were detected and Rf values for each of the TLC fractions were determined by the formula, Rf value= movement of solute from the origin/ movement of solvent from the origin.

Cell viability assay by MTT-[3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide)] Assay

Cytotoxic activity of compound against cancer cell lines was evaluated by MTT Assay. MTT is a yellow colored dye which is reduced into purple formazan crystal by the activity of mitochondrial succinate dehydrogenase enzyme in viable cells. HepG2, Jurkat, CHO and lymphocyte cells growing exponentially were trypsinized, cultured in a 96 well microtiter culture plates and were allowed to adhere for 24 h. Increasing concentration (5, 10 and 20 µg/ml) of the pigment fraction dissolved in DMSO were added to the well for 24 h. Controls were treated with only DMSO and the final concentration of 0.4%(v/v) of DMSO was maintained here. Evaluation of cytotoxicity was carried out using MTT assay, following the standard protocols **[9]**. The experiments were performed in triplicates and the absorbance was measured at 540nm using an ELISA plate reader. Percentage of inhibition was calculate by the formula, Percentage inhibition = (1-Optical Density of sample/Optical Density of control)×100.

Detection of bioactive fraction

Active compound separated in TLC was detected by bioassay guided fractionation. Partially purified samples obtained from the preparative TLC were allowed to evaporate and the concentration was maintained at 1mg/ml as a stock solution. Purified pigment fractions obtained, were tested at different concentrations for their cytotoxicity against HepG2, Jurkat, CHO and lymphocytes.

DNA fragmentation analysis

Cells in the exponential phase were incubated for 24 h. The cells were treated with the red pigment (SR6), and incubated for 24 h. Cells were collected by trypsination and genomic DNA was extracted from the cells, by treating them with 20 µL of RNAase A solution (incubated for 2 min at RT), 20 µL of proteinase K and 200 µL of lysis buffer A

(incubated in hot water bath at 70˚C for 10 min). After incubation, ice cold ethanol (200 µL) was added into the vial and centrifuged (10,000 rpm for 10 min). The precipitated DNA from the control and treated cells was dissolved in Tris-EDTA buffer (20 µL) and subjected to agarose gel electrophoresis.

Caspase 9 apoptotic assay

To check the apoptosis in mammalian cells, the caspase -9 activity was assessed using Caspase-9 Colorimetric Assay Kit (G Bio-sciences), which is a key indicator of apoptosis. The assay is based on detection of cleavage of a synthetic substrate, which has 7-amino-4-trifluromethyl coumarin (AFC) at the C-terminal. When liberated from the peptide, AFC produces an optical change that can be detected by either fluorescence or by colorimetric methods. Cells were treated with SR6 and incubated for 24h. The assay was performed by adding 50µL of 2X caspase assay buffer (containing DTT), 50 µL of cell lysates and 5 µL of 1mM AFC-conjugated substrate (50µM final concentration). Optical density (at 405 nm) was recorded at zero time point (t=0) in an ELISA plate reader and after every 15 min, OD was recorded until the measurements are significantly different from those at t=0. The percentage cytotoxicity was calculated using the following formula:

$$\text{Percentage activity of caspase} = (OD_{control/sample} - OD_{blank})/OD_{blank} \times 100$$

LDH analysis

Cellular cytotoxicity was assessed in the treated cells by performing the LDH cytotoxicity assay (kit from G Bioscience). This assay quantitatively measures an enzyme lactate dehydrogenase (LDH), which is released upon cell lysis. Cells were treated with the pigment (SR6) and incubated for 24 h. After incubation, cells were spinned followed by the addition of 10 µL of lysis buffer. Supernantant (50 µL) was collected to access the LDH release along with positive (only culture medium) and negative control (untreated cells). Reconstituted substrate mix (50 µL) was added to each well and incubated the assay plate at 37℃ for 20 min, protected from light following the addition of 50 µL of stop solution. Absorbance was recorded at 490nm and percentage cytotoxicity was calculated.

$$\text{Percentage cytotoxicity (\%)} = OD_{treated} - OD_{negative\ control}/OD_{positive\ control} \times 100$$

Statistical Analysis

Statistical significance was calculated by one-way analysis of variance (ANOVA). A value of $P<0.05$ was taken as statistically significant. All experiments were carried out in triplicates. The results were expressed as mean±standard error (SE) values.

RESULTS

The bacteria was identified as *Serratia marcescens* JGI 27 based on biochemical and morphological characterization by Bergey's classification of determinative bacteriology. They were subcultured once every 7 days and the pure cultures were maintained on nutrient agar slants.

Following extraction of the pigment by methanol, separation of crude extract into different fractions was carried out on TLC plates with the solvent system acetone and ethyl acetate (1:1). Nine different bands were obtained and chromatogram was detected by UV transilluminator (254 and 366 nm) (Figure 1). The partially purified fraction were tested for their cytotoxicity against HepG2, Jurkat, CHO cell lines and lymphocytes by MTT assay.

When HepG2 and Jurkat cells were treated with the different fractions obtained from preparative TLC, red pigmented fraction (SR6) showed best cytotoxic results and the percentage viability of HepG2 and jurkat cells was recorded as 45.9 and 47 % (after 24 h incubation) respectively at 20 µg/ml concentration. The IC50 value of SR6 on HepG2 and Jurkat cells were 16.08 and 16.54 µg/ml respectively (Figure 2 & 3, Table 1). Significant changes were not observed in the viability of HepG2 and Jurkat cells upon treatment with other fractions (results not shown). When CHO cells and lymphocytes were treated with SR6, the percentage viability was found in between 95-100% at all the selected concentrations and incubation time, indicating that SR6 is non toxic and comparatively least toxic to normal human cells.

Table 1: IC 50 values of SR6 on different cell lines

S.No.	Cell Lines	IC50 (µg/ml)
1.	HepG2	16.08
2.	Jurkat	16.54
3.	CHO	-
4.	Lymphocyte	-

When the DNA isolated from SR6 treated HepG2 cells was subjected to 0.8% agarose gel electrophoresis, a DNA smear characteristic of apoptotic DNA was observed in the cells treated with 20 µg/ml concentration of SR6. DNA from the control cells (untreated) appeared as a thick band indicating more number of viable cells and the presence of intact DNA (Figure 4).

Apoptotic induction by SR6 was determined by the activity of caspases, which is a principal biochemical hallmark in apoptotic signalling. Caspase-9 in one of the major effector caspases that plays a significant role in the implementation of apoptotic cell death. There was a

significant increase in the activity of caspase-9 in the HepG2 and Jurkat cells treated with SR6 after 24 h of incubation. The percentage increase in the caspase activity in HepG2 and jurkat cells was found to be 67.85 (after 45 min) and 65.4% (after 60 min) respectively, indicating the apoptotic induction and there was around 5-6 fold increase of caspase activity in the treated HepG2 and Jurkat cells as compared to that of control cells (Figure 5).

The cellular cytotoxicity was assessed by LDH release from the apoptotic cells. The percentage cytotoxicity of HepG2 and Jurkat cells was found to be 68.36 and 59.07% as compared with that of positive control(1% Triton-X 100) when treated with 20 µg/ml of SR6, indicates that SR6 is cytotoxic and released LDH when added to HepG2 and Jurkat cells (Figure 6).

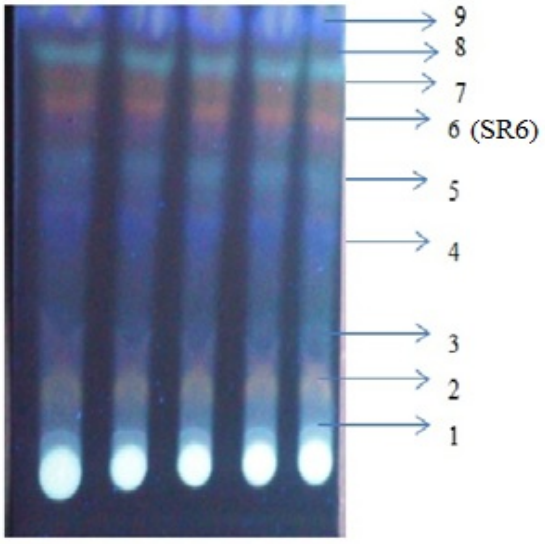

Fig-1: Thin layer chromatography of separated fractions (1-9) under UV transilluminator. Fraction 6- SR6 (IC_{50}= < 20 µg/ml)

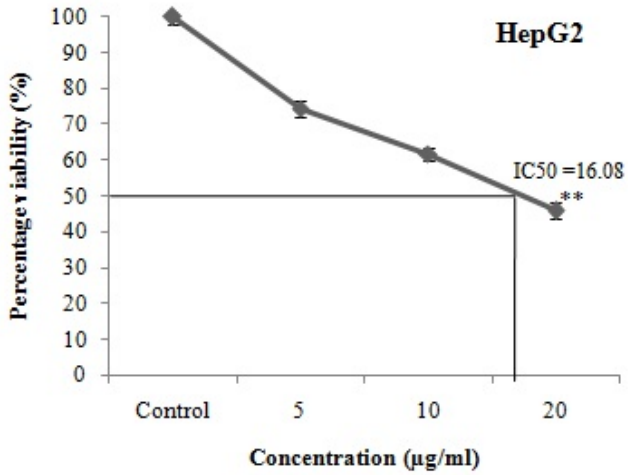

Fig-2: Effect of different concentrations of SR6 from *S.marcescens* on the percentage viability of HepG2 cells and calculation of IC50 value. Vertical bars indicate SE of the mean for n = 3. ** indicates p< 0.001 as compared to the control.

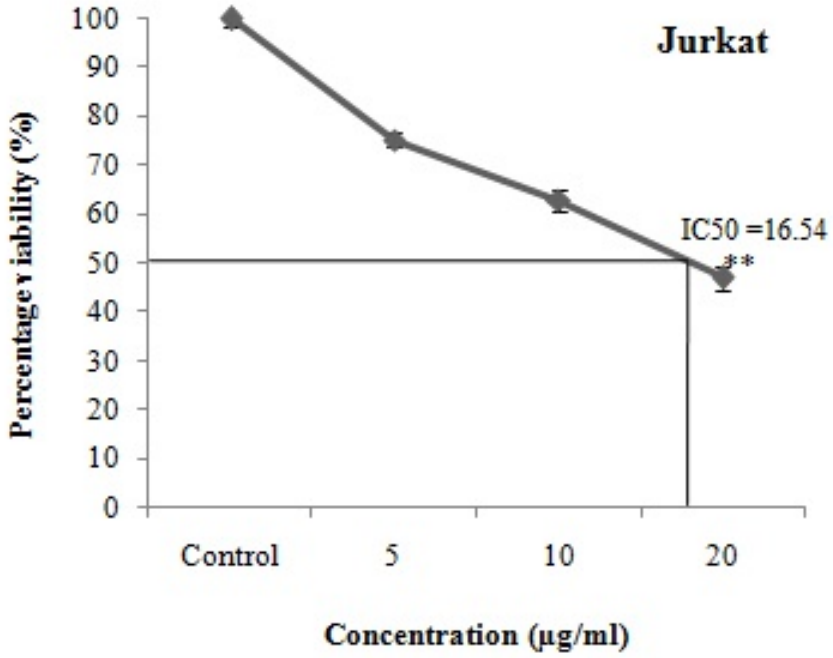

Fig-3: Effect of different concentrations of SR6 from *S.marcescens* on the percentage viability of jurkat cells and calculation of IC50 value. Vertical bars indicate SE of the mean for n = 3. ** indicates p< 0.001 as compared to the control.

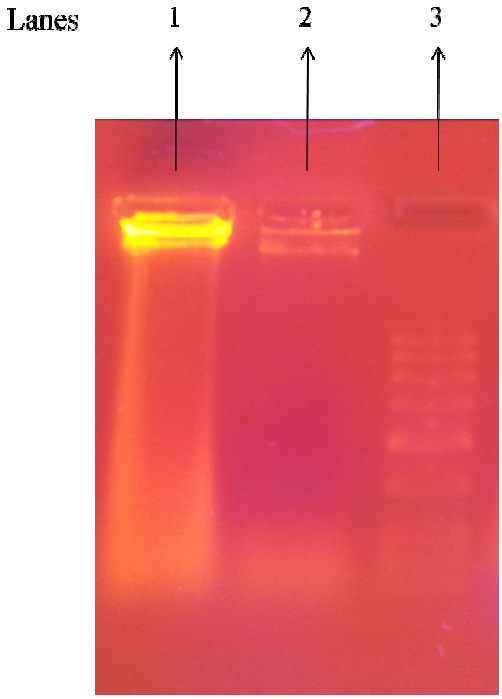

Fig-4: DNA fragmentation analysis of HepG2 cells treated with the SR6, Lanes 1. DNA from the HepG2 cells treated with 20µg/ml of SR6 2. DNA from the control HepG2 cells 3. DNA ladder

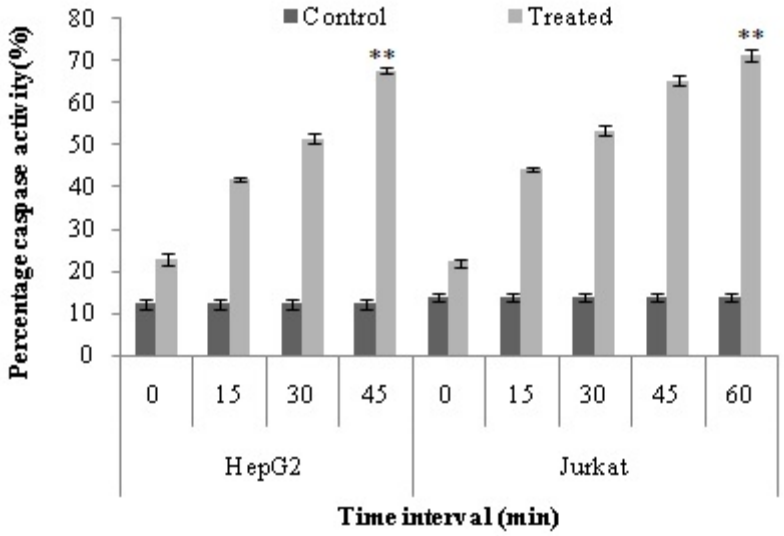

Fig-5: Percentage caspase activity between Control and SR6 treated cells with respect to time interval. Vertical bars indicate SE of the mean for n = 3. ** indicates p< 0.001 as compared to the control

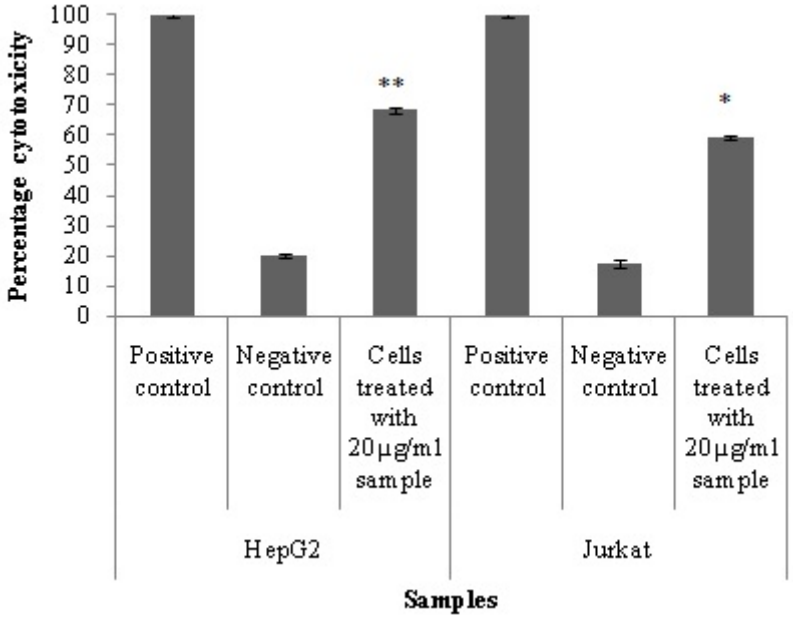

Fig-6: Percentage cytotoxic effects of SR6 from *S.marcescens* determined by LDH release. Vertical bars indicate SE of the mean for n = 3. *indicates significance with p< 0.05 and ** indicates p< 0.001 as compared to the control.

DISCUSSION

Apoptosis or programmed cell death, is a biological process that is involved in the action of several cancer chemotherapeutic agents and the apoptotic hallmark corresponding to cell death are DNA fragmentation, cell shrinkage, formation of apoptotic bodies and so on. The prodiogiosin family of red pigments is produced by *Serratia* species and was found to exhibit cytotoxic and anticancerous activities. In this study, we

assessed the cytotoxic potential of the red fraction (SR6) from *S.marcescens* JGI 27 on the liver cancer cell line and leukemia cell line. Our findings demonstrated that SR6 exhibited promising anticancer potential against HepG2 and jurkat cell lines and it is non toxic to CHO and lymphocyte cells. As it has strong antiproliferative effect, this fraction was further analysed and the cytotoxicity was confirmed by various *in-vitro* assays. SR6 was purified by thin layer chromatography with the solvent system acetone and ethyl acetate (1:1). The percentage viability of HepG2 and Jurkat cells were declined to 45.9 and 47 % and IC_{50} value of SR6 was found to be at 16.08 and 16.54 µg/ml concentration respectively. Caspase-9 which is an important signalling molecule is a caspase protein that plays central role in the execution phase of apoptosis. In our study, SR6 induced apoptosis in the treated cells (HepG2 and jurkat), as manifested by 5-6 fold increase in the percentage caspase activity as compared to that of the control cells. Futhermore, the percentage cytotoxicity in the HepG2 and jurkat cells were found to be 68.36 and 59.07%, when treated with SR6, which indicates the release of LDH upon cell lysis which is a marker of apoptosis. Apoptotic induction of cell death in a SR6 treated cells was again confirmed by DNA fragmentation analysis, where we observed DNA smear and decreased concentration of treated cells. Hence, it is certain that the SR6 induced apoptosis in liver cancer cell line and leukemia cells. However, further chemical analysis of the compound to identify the chemical group and animal studies are required to prove this.

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