Journal of Research in Biology

An International Scientific Research Journal

Original Research

Effect of extract of *Bacillus megaterium* 9554 on oral cancer cell line SCC131

Authors:
Vipin Mohan Dan[1],Vinitha Richard[2], Asha Nair[2], Palpu Pushpangadan[1], Varughese George[1], Ajit Varma[3] and Radhakrishna Pillai[2].

Institution:
1. Amity Institute of Herbal and Biotech Product Development, Trivandrum, Kerala.

2. Department of Molecular Medicine, Rajiv Gandhi Centre for Biotechnology, Trivandrum, Kerala.

3. Amity Institute of Microbial Technology, Amity University, Noida, Uttar Pradesh.

ABSTRACT:

A microbe, identified as *Bacillus megaterium* 9544 was isolated from the soil sample collected from Manali, Himachal Pradesh. The bacterial broth extract had the presence of compound(s) that induced apoptotic like features on oral cancer cells, like morphological changes, condensation of nucleus and detachment of cancer cells from the surface of culture vessel. The extract did not cause any kind of changes in mitochondrial membrane potential as evidenced from flow cytometric analysis and did not cause fragmentation of nuclear DNA. This particular compound(s) which was present in the bacterial extract seemed to induce only a weak apoptotic signal that did not lead to complete progression of apoptosis.

Keywords:
Apoptosis, Flow cytometry, *Bacillus,* Cancer.

Corresponding author:
Vipin Mohan Dan and Ajit Varma.

Phone No:
(0471) 2432138.

Fax
+91 120 2431268

Email:
vipindan@yahoo.co.in

Web Address:
http://jresearchbiology.com/documents/RA0262.pdf.

Article Citation:
Vipin Mohan Dan,Vinitha Richard, Asha Nair, Palpu Pushpangadan, Varughese George, Ajit Varma and Radhakrishna Pillai.
Effect of extract of *Bacillus megaterium* 9554 on oral cancer cell line SCC131.
Journal of Research in Biology (2012) 2(5): 523-528

Dates:
Received: 03 Jul 2012 **Accepted:** 18 Jul 2012 **Published:** 25 Jul 2012

INTRODUCTION

Soil bacteria have been a treasure of many valuable natural products ranging from antibiotics, anticancer compounds to other secondary metabolites of industrial and agricultural importance. Microbes have a perplexing relation with humans, from mutual beneficial associations in the human body, to the fatal diseases that other members cause and the counteractive compounds yet other family members produce to ward-off or cure such infections. Cancer is one of the diseases where intensive research is carried out globally in search of a cure. Soil bacteria have contributed immensely in producing many clinically viable anticancer drugs. Actinomycin D is the oldest microbial metabolite used in cancer therapy. Its relative, actinomycin A, was the first antibiotic isolated from actinomycetes and was obtained from *Streptomyces antibioticus* by Waksman and Woodruff (1941).Anthracyclines rank among the most effective anticancer drugs ever developed (Weiss, 1992). They are effective against most types of cancer than any other class of chemotherapy agents. They are used to treat a wide range of cancers, including leukemias, lymphomas, and breast, uterine, ovarian and lung cancers. Anthracyclines act by intercalating DNA strands, which result in a complex formation that inhibits the synthesis of DNA and RNA. It also triggers DNA cleavage by topoisomerase II, resulting in mechanisms that lead to cell death (Weiss 1992). Among the *Bacillus* species, a class of protein called Parasporins that come under *Cry* proteins produced by *Bacillus thuringiensis* had shown cytocidal activity against human cancer cell lines (Mizuki *et al*., 2000). In another study *Bacillus vallismortis* BIT-33 produced a compound of anticancer nature that was effective against various colon cancer cell lines (Jeong *et al*., 2008). The present study involves the investigation of culture extracts of *Bacillus megaterium* 9554 for presence of compound(s) with anticancer potential.

MATERIALS AND METHODS

Cancer cell line

Human oral cancer cell line SCC131 was procured from National Centre for Cell Sciences (NCCS, Pune). The cell line was grown in DMEM (Dulbecco's Modified Eagle Media) medium supplemented with 10 percent FBS (Fetal Bovine Serum) in a 5 percent CO_2 incubator at 37°C. Antibiotics penicillin (100µg/mL) and streptomycin (100µg/mL) were also added into the medium to avoid any bacterial contamination.

Microorganism

The bacterial strain used in this study was *Bacillus megaterium* 9554, which was previously isolated from soil samples collected from Manali, Himachal Pradesh. The bacterial culture was routinely maintained on Nutrient agar slants. The organism was subcultured every month. The bacterial isolate was identified based on morphological, physiological and biochemical characteristics as described in Bergey's manual of determinative bacteriology (Holt *et al*., 1994).

Bacterial extract

The bacterial culture was grown in nutrient broth at 150rpm, 37°C for 48 h. The broth was centrifuged and the supernatant was filtered to remove bacteria. The pH of supernatant was adjusted to 2 with Conc. HCl and then centrifuged at 16,000g, 10 min at 4°C. The precipitate obtained was dissolved in Dimethyl sulphoxide: DMEM (50:50), pH was maintained at 8,filtered through 0.2µm filter and kept at -20°C.

Microscopic analysis and MTT assay

Cancer cells were seeded in 96-well plates at a density of 5000 cells per well. The attached cells were incubated for 24 h and then treated with different concentrations (25µg, 50µg, 100µg, 150µg, 200µg, 300µg and 400µg per ml of DMEM medium) of bacterial extract, and were then incubated for 24 and

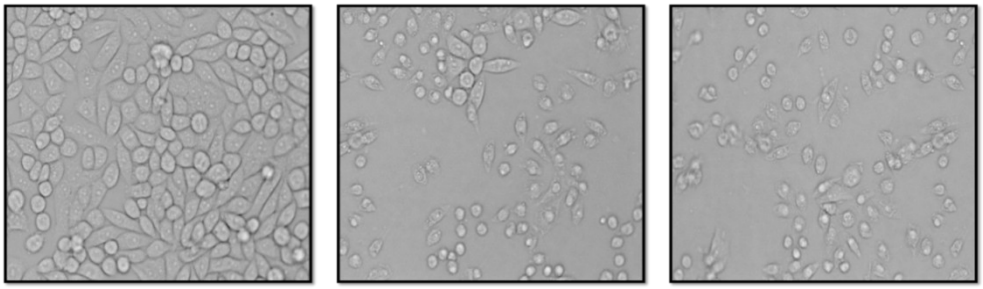

Figure 1: Effect of extract treatment on oral cancer cell line. Changes in cell morphology were observed on treatment with extract.

48 h. The cells were visually analyzed with microscope for morphological changes. The cell viability was determined with 20µl of MTT solution (5 mg/ml). The plates were incubated for 4 h at 37°C. DMSO was used to dissolve the formazan crystals that formed and soluble formazan product was spectrophotometrically quantified using an ELISA reader at 575 nm.

Nuclear Staining with Hoechst 3342

Trypsinized cells were seeded into 12 well plates at 10,000 cells per well and kept for 24 h incubation. The spent media was aspirated and cells were rinsed with PBS, different concentrations of the bacterial extract were added to respective wells. Plates were placed in carbon dioxide incubator. After 24 and 48 h each well was incubated with Hoechst 3342 (5 µl/mL) for another 15 min at 37°C in carbon dioxide incubator. The samples were analyzed through fluorescence microscope.

Mitochondrial membrane potential analysis

MitoProbe™ JC-1 Assay Kit procured from Life technologies was used for analyzing mitochondrial membrane potential of control and treated oral cancer cell line by flow cytometry. Cells were cultured in 12 well plates at a density of 5 x 10^5 cells per well and incubated in CO_2 incubator for overnight at 37°C. The cells were treated with bacterial extract and untreated cells were taken as control and incubated for 48 h. After the incubation period 100 µl of JC-1 staining solution was added per mL of culture medium to each well of the plate. The cells were incubated in a CO_2 incubator at 37°C for 15-30 min. The cells were harvested from each well and were analyzed using flow cytometry.

DNA Fragmentation assay

Oral cancer cells were plated in culture plates at a density of 1x10^5 /ml and then treated separately with different concentrations of bacterial extract (150µg/ml and 300µg/ml) and curcumin (100µg/ml) for 48 h. DNA was extracted by the method described by Mori *et al*., (2001). DNA fragmentation was analyzed on agarose gel electrophoresis.

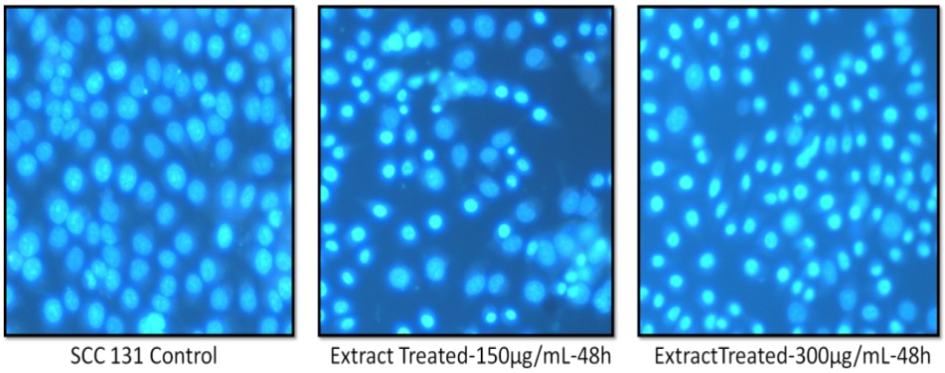

Figure 2 : Nuclear staining with Hoechst 3342 assay. Condensation of nucleus (intense bright fluorescence) was observed in the two extract treatments as compared to control cancer cells at 48h.

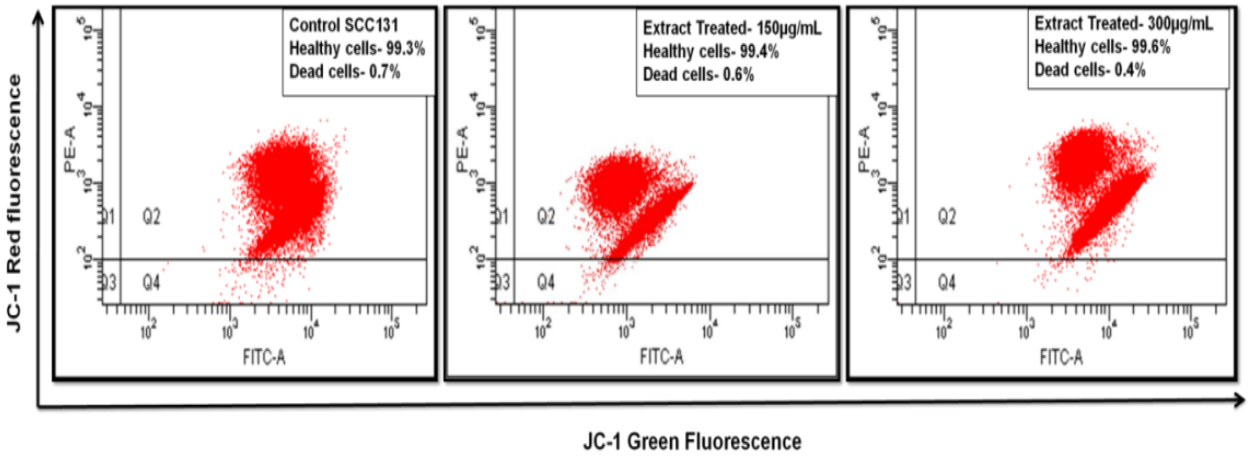

Figure 3: Mitochondrial membrane potential analysis of control and extract treated SCC131 oral cancer cell line at 48h.Viable cells are seen in Quarter Q2 while dead cells are seen in Quarter Q4.The percent of viable cancer cells remained above 99% in two treatments as well as control.

RESULTS AND DISCUSSION

Bacillus megaterium 9554 was identified based on morphological, biochemical and physiological characteristics (Table 1) as per the Bergey's manual of determinative bacteriology (Holt *et al*., 1994). The treatment with extract caused morphological changes in cancer cells as evidenced from microscopic analysis, the cells lost their striated shape, attained a rounded morphology and detached from surface of culture vessel (fig. 1). Initial analysis with MTT assay revealed that morphologically redefined cells were viable as it utilized MTT thereby signifying the presence of intact mitochondria with mitochondrial dehydrogenase enzyme responsible for cleaving the yellow MTT to blue formazan crystals. Two concentrations of the bacterial extract, 150 µg and 300µg, that produced effective morphological change in more than 50% of the cells were selected for further study. Hoechst staining revealed that the oral cell line showed condensation of nucleus on treatment with bacterial extract (fig. 2). During the early process of apoptosis, cell shrinkage and pyknosis are visible by light microscopy (Kerr *et al*., 1972). Pyknosis is the result of chromatin condensation and this is the most characteristic feature of apoptosis (Elmore 2007).

Mitochondrial membrane potential assay, revealed that the bacterial extract had no compounds of anticancer nature, with the two treatments and control registering, above 99 percent healthy viable cells with no changes in mitochondrial membrane potential (fig. 3). DNA fragmentation assay confirmed that the apoptotic features initially induced by bacterial extract did not lead to fragmentation of cell's DNA while treatment with curcumin (positive control) gave the fragmented DNA ladder pattern (fig. 4). The cells even though initially showed apoptotic features like morphological changes, detachment from culture vessel and condensation of

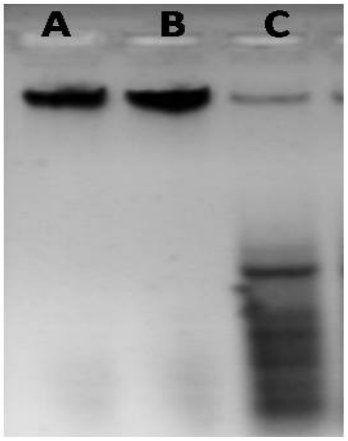

Figure 4: DNA Fragmentation assay. A: Extract (150µg/ml) treated SCC131 DNA; B: Extract (300µg/ml) treated SCC131 DNA; C: Curcumin (100µg/ml) treated, fragmentation of SCC131 DNA was caused only by curcumin.

Table1: Morophological, biochemical and physiological characteristics of *Bacillus megaterium* 9554;+: positive; -: negative; (+): Weak positive

Tests	
Colony morphology	
Gram's reaction	+ve
Cell shape	rods
Size (µm)	2-4
Spores	+, central, oval
Motility	-
Physiological tests	
Growth Temperatures	4°C to 50°C
pH range	7 – 9
NaCl (%)	2 - 10
Biochemical tests	
MacConkey agar	-
Indole test	-
Methyl red test	+
Voges Prokauer test	-
Citrate utilization	+
H_2S production	-
Caesin hydrolysis	-
Esculin hyrolysis	-
Gelatin hyrolysis	+
Starch hyrolysis	+
Nitrate reduction	(+)
Catalase test	+
Oxidase test	+
Arginine dihydrolase	+

nucleus on extract treatment eventually did not progress to complete apoptosis. This cell survival phenomenon may be due to weak apoptotic signals, as per Hoeppner *et al*., (2001), weak pro-apoptotic signals can enhance cell survival. It can also be hypothesized that the bacterial extract may have presence of compound(s) that can affect the anchoring proteins of cancer cells and also create a slightly stressful environment for the cancer cells.

CONCLUSION:

The culture broth extract of *Bacillus megaterium* 9554 had the presence of compound(s) that produced weak apoptotic signals or created a slightly stressful environment that led to morphological changes, condensation of nucleus and detachment of oral cancer cell line SCC131. Further investigation is required to disclose, the scientific reason that causes, such initial apoptotic like features in the cancer cells but which does not progress to a complete apoptotic pathway.

ACKNOWLEDGEMENT

The author would like to thank CSIR, India for providing Research associate fellowship. The author would like to express sincere gratitude to Prof Radhakrishna pillai, Director, Rajiv Gandhi Centre for Biotechnology (RGCB), Kerala for providing research facilities. Special thanks to Mrs Indu Ramachandran, Senior Technical Officer, RGCB for helping with flow cytometry.

REFERENCES

Elmore S. 2007. Apoptosis: A Review of Programmed Cell Death. Toxicol Pathol. 35:495-516.

Hoeppner DJ, Hengartner MO and Schnabel R. 2001. Engulfment genes cooperate with ced-3 to promote cell death in Caenorhabditis elegans. Nature 412:202-6.

Holt JG, Krieg NR, Sneath PHA and Staley JT. 1994. Bergey's Manual of Determinative Bacteriology. Nineteenth edition, Williams and wilkins company, Baltimore, MD, USA 255-273.

Jeong SY, Park SY, Kim YH, Kim M and Lee SJ. 2008. Cytotoxicity and apoptosis induction of *Bacillus vallismortis* BIT-33 metabolites on colon cancer carcinoma cells. J. of Appl. Microbio., 104:796-807.

Kerr JF, Wyllie AH and Currie AR. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239-57.

Mizuki E, Park YS, Saitoh H, Yamashit S, Akao T, Higuchi K and Ohba M. 2000. Parasporin, a human leukemic cell-recognizing parasporal protein of

Bacillus thuringiensis. Clin Diagn Lab Immunol. 7:625-634.

Mori H, Niwa K and Zheng Q. 2001. Cell proliferation in cancer prevention; effects of preventive agents on estrogen-related endometrial carcinogenesis model and on an in vitro model in human colorectal cells. Mutat Res, 480-1, 201-7.

Waksman SA and Woodruff HB. 1941. *Actinomyces antibioticus*, a new soil organism antagonistic to pathogenic and non-pathogenic bacteria. *J. Bacteriol.* 42: 231-249.

Weiss RB. 1992. The anthracyclines: will we ever find a better doxorubicin? *Semin Oncol*. 19:670-686.