INTERNATIONAL RESEARCH JOURNAL OF PHARMACY
www.irjponline.com
ISSN 2230 – 8407

Research Article

CYTOTOXIC ACTIVITY OF PROBIOTIC *BACILLUS SUBTILIS SK09* AGAINST HUMAN COLON CANCER CELL LINE (HT-29)

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Article Received on: 09/03/17 Approved for publication: 22/04/17

DOI: 10.7897/2230-8407.080458

ABSTRACT

Probiotics are provided as nutraceutical supplements to aid in proper digestion of lactose rich dairy products and act as enhancers of prophylaxis. The probiotic organism *Bacillus subtilis SK09*, isolated from dairy effluents, is capable of metabolizing lactose and sporulating under stressed conditions. Its metabolite analysis profile confirms that it is a novel strain, which produces anti-carcinogenic compounds such as 8-Octadecenoic acid, methyl ester; (2,7-Diphenyl-1-6dioxopyridazino(4,5:2'3') pyrrolo(4',5'-d)pyridazine; Octadec-9-enoic acid; Hexadecanoic acid, methyl ester; and 4-Piperidineaceticacid, 1-acetyl-5-ethyl-2-(3.(2.hydroxyethyl)-1H-indol-2-yl)-a-methyl, methyl ester. The present study is focused on the anti-cancerous activity of *Bacillus subtilis SK09* on HT-29 cell line (Human colon cancer cell line) confirmed by 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay. The *in vitro* cytotoxic assay of probiotic biomass showed the significant cytotoxicity with IC50 of 7.8µg/ml. Therefore, *Bacillus subtilis SK09* could be served as probiotic organism with anti-cancerous therapeutic applications.

Keywords: Probiotics, *Bacillus subtilis SK09*, HT-29 cell line, MTT assay.

INTRODUCTION

Cancer is a class of disease, in which cells grow abnormally in an uncontrolled manner. 13% of the world's population is affected by cancer and it is regarded as the second most common cause of death in humans[1,2]. In recent years, great progress has been made in cancer detection and treatment[3]. Cancer detection is a very crucial step in the diagnosis of any type of cancer, as it deals mainly with prevention and its control. Even though, histopathology is considered as the typical method for cancer diagnosis, recent techniques which include SELDI-TOF mass spectrometry, MRI, CT, MRS, IHC, PCR, flow cytometry, FISH, CSH and microarray, ultrasonography serve as a significant breakthrough in diagnosis of cancer[4,2]. There are many types of cancers, which include breast cancer, lung cancer, blood cancer, stomach cancer, colon cancer, prostate cancer etc.

Among men and women, colon cancer acts as the third most common type of cancer which is influenced by genetic, physiological and environmental factors. Risk factors such as age, eating habits, physical activity, alcohol consumption, smoking, nutritional status, presence of polyps, cancer history of self and family, cases of ulcerative enterocolitis and chronic constipations, may be the reasons for the formation of colon cancer and even ingestion of a high-fat diet and high animal protein can lead to colon cancer. There is a high possibility of cancer risk in large intestine than in the small intestine because of the high bacterial density. The comparatively large number of bacteria present in the colon show higher fermentation ratios and proliferation rates[5,6]. By manipulating metabolic, immunologic, and protective functions in the colon, lactic acid bacteria play a pivotal role in obstructing colon carcinogenesis[7]. Pre-cancerous polyps can be eliminated by performing screening and detection in the previous stage of colorectal cancer[8]. Colon cancer risks can be prevented by probiotics as specified by the *in vitro* and *in vivo* studies. Reduction of H_2O_2 levels that leads to tumour progression can be controlled by introducing an effective strain namely *Lactococcus lactis*, which is investigated in DMH induced murine model[9].

Probiotics are live microorganisms isolated from human and intestinal tracts of animals which when administered in adequate amounts confer a health benefit on the host[10,11]. They are used as food and food supplements which are in the form of capsule or tablet[12]. Several probiotic strains help to balance intestinal micro-flora by preventing malfunction of the gastrointestinal tract and act as a supplement to improve the digestion of dairy products have been reported[13,14]. The experimental probiotic strain *Bacillus subtilis SK09* has the ability to ferment lactose, produce antimicrobials and also can survive at the internal gut temperature by expressing its probiotic activity[15-17]. In the current study, a complete analysis of its metabolite profile has been made to ascertain its probiotic value towards curing gastro intestinal disorders and cytotoxic activity against colon cancer.

MATERIALS AND METHODS

Culture propagation

Duplicates of 100ml Lactobacillus MRS Broth was prepared in a 500ml Erlenmeyer flask with sterile distilled water and adjusted to pH 7.0, then sterilized in an autoclave at 121°C and 15psi. The broth was cooled in a laminar air chamber and inoculated with 5% v/v of primary inoculum of *Bacillus subtilis SK09*. These flasks were placed in a rotary shaker maintained at 32°C for 24 hours at150rpm.

Active principle isolation

After 24 hours, the microbial cultures were centrifuged in sterile 50ml round bottomed centrifuge tubes at 6000 rpm for 15 min at 4°C. The cell free supernatant was collected in sterile 100ml polystyrenes storage vials and stored at 4°C for future use. To the cell pellet 5ml of phosphate buffer was added and lysed by ultrasonication.

Product extraction

The active principle isolation was carried out by precipitation reaction using salting-out technique. Ammonium sulphate salt was added very slowly to supernatant until it gets to 60% saturation at 4°C. It was then centrifuged at 6000rpm for 20 min at 4°C. The final precipitate (extracellular protein fraction) and the supernatant (extracellular non-protein fraction) were carefully collected and stored at 4°C for further analysis. Same procedure was adopted for cell lysate solution and the final solution of intracellular protein fraction of cell lysate was collected and stored at 4°C for further analysis.

GC-MS analysis

To find out the bioactive compounds present in the precipitate (extracellular protein fraction), the supernatant (extracellular non protein fraction) and the lysate (intracellular protein fraction) of probiotic *Bacillus subtilis SK09*, GC-MS (JEOL GC MATE II) analysis was done with an HP 5 MS column at the front inlet temperature at 220°C. High purity helium was used as a carrier gas at a constant flow rate of 1ml/min. Injection volume of 1µl was employed with the addition of methanol and chloroform as a solvent in 3:1 ratio. Ion chamber temperature was at 250°C. Here Quadrupole double focusing mass analyzer was used. The oven temperature was programmed from 50°C to 250°C at10°C/min. Gas chromatography interface temperature was at 250°C.

For the MS, electron impact ionization was carried out at 70 eV; scan range was found to have 50 to 600 amu. Identification of bioactive compounds and mass spectra comparison was performed using the NIST Ver.2005 MS data library.

Cell line culture medium

Cell line studies were done using HT-29 (Human colon cancer cell line) procured from National Centre for Cell Sciences, Pune, India. The cells were maintained in Minimal Essential Medium (Hi Media Laboratories, India) supplemented with 10% Fetal Bovine Serum (Cistron laboratories), penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37°C.

***In vitro* assay for anticancer activity (MTT assay)**

In 24-well plate, the HT-29 cells of concentration 1×10^5/well were plated and were incubated at 37°C with 5% CO_2. Once the cells reach the required confluence, the different concentrations of the sample were added and were incubated at 37°C, with 5% CO_2 for 24 hrs. After 24 hrs, the samples were removed and then washed with phosphate-buffered saline (pH 7.4). Then 100µl of 0.5% of MTT 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide (Sisco research laboratory chemicals, India) was added in each well and then incubated for 4 hrs.

Once the incubation period is over, 1ml of DMSO was added to all the wells and their absorbance was measured at 570nm with UV –Visible spectrophotometer using DMSO as blank. A graph was plotted with percentage (%) cell viability at y-axis and concentration of the sample in x-axis, using these absorbance data. With this graph the concentration required for 50% inhibition (IC50) was determined. Here both the cell and sample control were included in each assay to compare the full cell viability.

Table 1: Identified compounds from methanol - chloroform extract of *Bacillus subtilis SK09*

Compound name	Retention time	Mol. Formula/ Weight	Peak area (%)	Bioactivity/References
8-Octadecenoic acid, methyl ester	18.9	$C_{19}H_{36}O_2$ 296.4879	35.51	Antimicrobial [19] and anti-carcinogenic activity [20]
2,7-Diphenyl-1-6 dioxopyridazino (4,5:2'3') Pyrrolo (4',5'- d) pyridazine	22.97	$C_{20}H_{13}N_5O_2$355.34952	15.79	Cytotoxic against human cancer cell line [21]
Octadec-9-enoic acid	19.75	$C_{18}H_{34}O_2$ 282.46	18.46	Anticancer & antileukemic activity [19, 22-24]
Hexadecanoic acid, methyl ester	17.18	$C_{17}H_{34}O_2$ 270	20.25	Antimicrobial [25] and anticancer [26] activity
4-Piperidineacetic acid ,1-acetyl 5-ethyl-2-(3.(2.hydroxyethyl)-1H-indol-2-yl)-a-methyl, methyl ester	22.97	$C_{13}H_{18}N_2O_2$ 234.29422	25.13	Antimicrobial activists [27] and Anticancer activity [28]

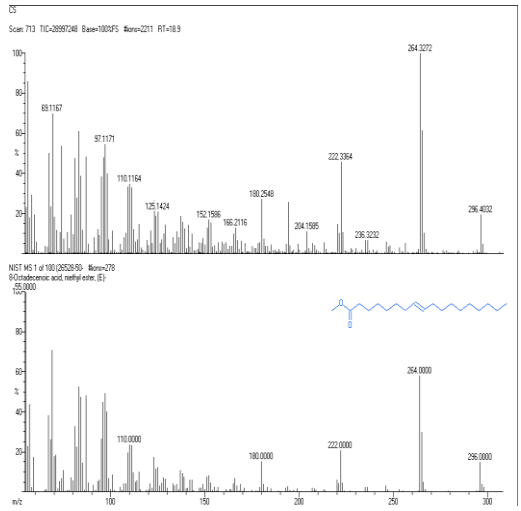

Figure 1. Mass spectrum of 8-Octadecenoic acid, methyl (RT: 18.9) from extracellular non-protein fraction *Bacillus subtilis SK09*

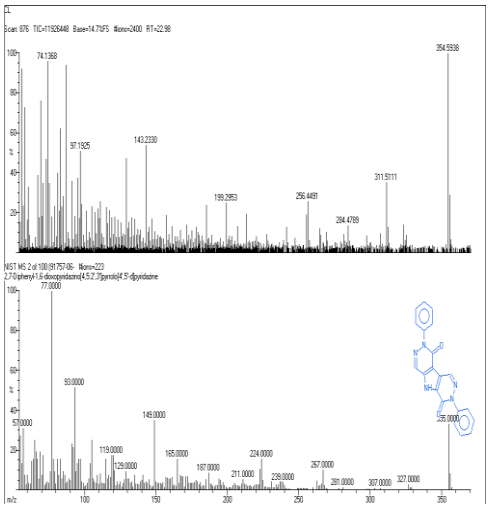

Figure 2: Mass spectrum of 2, 7-Diphenyl-1-6 dioxopyridazino (4, 5:2'3') pyrrolo (4',5'.d) pyridazine (RT:22.98) from extracellular non-protein fraction *Bacillus subtilis SK09*

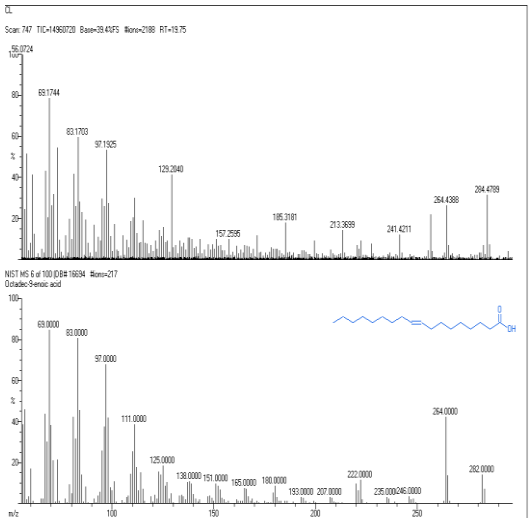

Figure 3: Mass spectrum of octadec-9-enoic acid (RT: 19.75) from intracellular protein fraction of *Bacillus subtilis SK09*

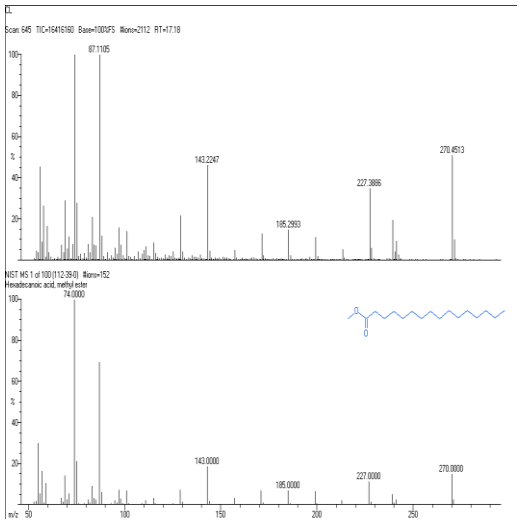

Figure 4: Mass spectrum of Hexadecanoic acid, methyl ester (RT: 17.18) from intracellular protein fraction of *Bacillus subtilis SK09*

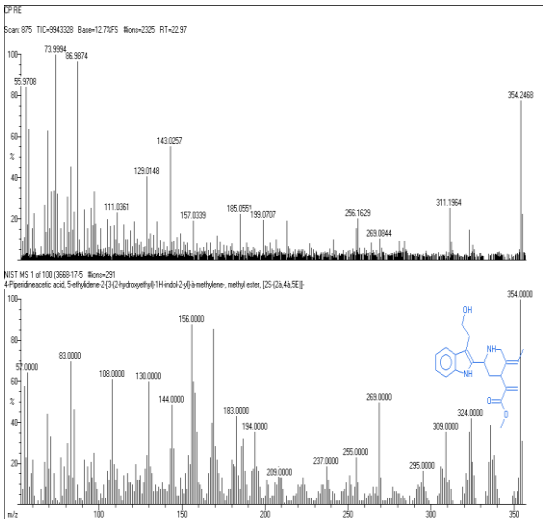

Figure 5: Mass spectrum of 4-Piperidineacetic acid, 1-acetyl-5- ethyl-2- (3.(2.hydroxyethyl)-1H-indol-2-yl)-a-methyl methyl ester (RT: 22.97) from extracellular protein fraction of *Bacillus subtilis SK09*

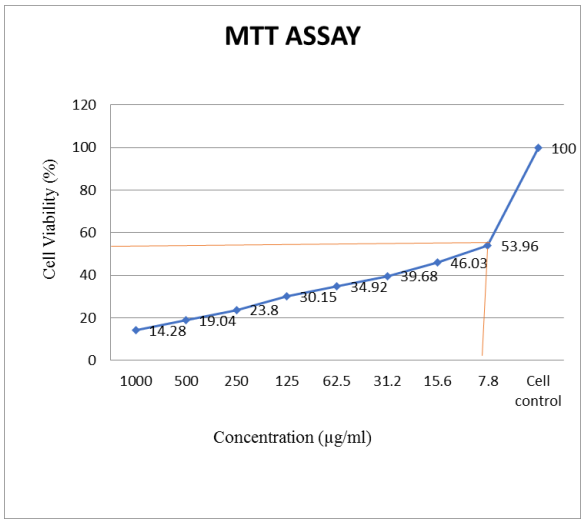

Figure 6: MTT assay *Bacillus subtilis SK09* against HT-29 colon cancer cell line

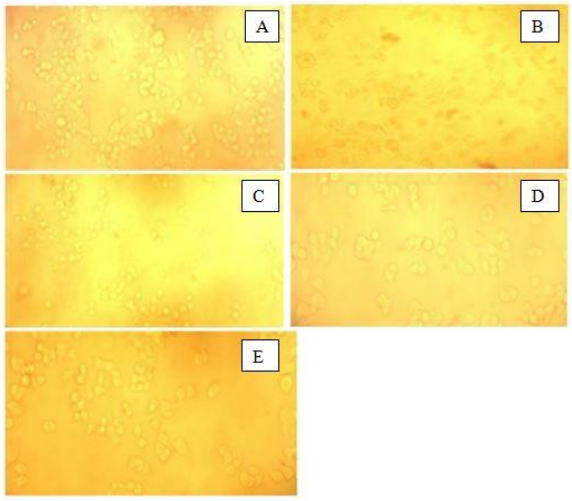

Figure 7: HT-29 cell line treated with various concentrations of probiotic *Bacillus subtilis SK09* (A) Toxicity-1000 µg/ml (B) HT-29 cell line control (C) Toxicity-62.5 µg/ml (D)Toxicity-15.6 µg/ml (E) Toxicity-7.8 µg/ml (IC50)

RESULTS AND DISCUSSIONS

GC-MS analysis

The GC-MS spectral results of extracellular and intracellular metabolites and comparison of results with library search enabled the identification of five anti-carcinogenically active compounds:
(i) 8-Octadecenoic acid, methyl ester;
(ii) 2, 7-Diphenyl-1-6dioxopyridazino (4, 5:2'3') pyrrolo(4', 5'-d)pyridazine;
(iii) Octadec-9-enoic acid;
(iv) Hexadecanoic acid, methyl ester;
(v)4-Piperidineaceticacid,1-acetyl-5-ethyl-2-(3(2.hydroxyethyl)-1H-indol-2-yl)-a- methyl, methyl ester.

The details of identified compounds are presented in Table 1.

Two major bioactive compounds present in the extracellular non-protein fraction were, a fatty acid ester namely 8-Octadecenoic acid, methyl ester (Figure.1) at the retention time of 18.9 was proven to have antimicrobial and anti-carcinogenic activity with a peak area of 35.51 %[19, 20] and 2, 7-Diphenyl-1-6 dioxopyridazino (4,5:2'3') pyrrolo (4', 5'- d) pyridazine (Figure. 2) at the retention time of 22.97 was found to have cytotoxicity against human cancer cell line with a peak area of 15.79 % [21].

Two other compounds, which was found in intracellular protein fraction were Octadec-9-enoic acid (Figure.3), a mono unsaturated omega-9-fatty acid (oleic acid) at the retention time of 19.75 was found to have anticancer and moderate anti-leukemic activity with a peak area of 18.46 % [19, 22-24] and a fatty acid namely Hexadecanoic acid, methyl ester (Figure.4) at the retention time of 17.18 were found to have antimicrobial activity and also acts as anticancer agent with a peak area of 20.25% [25,26]. The last compound, which was present in the extracellular protein fraction namely 4-Piperidineacetic acid, 1-acetyl-5-ethyl-2-(3.(2.hydroxyethyl)-1H-indol-2-yl)-a-methyl methyl ester (Figure. 5) at the retention time of 22.97 was found to have antimicrobial activity and acts as anticancer agent with a peak area of 25.13 % [27, 28].

Anticancer activity

The cytotoxic activity for probiotic biomass of *Bacillus subtilis SK09* against HT-29 colon carcinoma cells line was analyzed by using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) assay. The percentage of cells surviving was counted according to the optical absorption value of the HT-29 cells which was exposed to various biomass concentrations of *Bacillus subtilisSK09*. The results showed that cell viability dropped by 53.96% when treated with 7.8 µg/ml of probiotic biomass on HT-29 colon cancer cell line (Figure.6).

These results confirms that the IC50 value of probiotic biomass of *Bacillus subtilis SK09* against HT-29 human colon cancer cell line was 7.8µg/ml, when compared to the previous findings of marine *Bacillus vallismortis* BIT-33 and *Bacillus circulans* DMS-2 with 10 µg/ml and 120 µg/ml respectively[29,30]. Hence it is proved that *Bacillus subtilis SK09* was found to have better anticancer activity with cell viability of 53.96 % of HT-29 human colon cancer cell line. Thus the novelty of probiotic strain of *Bacillus subtilis SK0 9* has been proved for its effective and potential cytotoxicity.

CONCLUSION

A probiotic strain of *Bacillus subtilis SK09* has been analysed for its metabolites, both at intra and extracellular level. The metabolite screening revealed the presence of five pertinent bioactive compounds with anti-carcinogenic properties. Hence this probiotic strain was checked for its anti-cancerous activity on HT-29 (human colon cancer cell line). MTT assay confirmed for its potential cytotoxicity with IC50 value of 7.8µg/ml, which is significantly high. Our results suggest that the probiotic strain of *Bacillus subtilis SK09* could be initiated as new drug molecules for anticancer and therapeutic applications. Further studies to elucidate its anti-carcinogenic mechanism will be undertaken in the future.

ACKNOWLEDGEMENTS

Authors acknowledge the faculty of Indian Institute of Technology Madras (Sophisticated Analytical Instrument

Facility), Chennai for GC-MS analysis and M/s. Life Tech, Chennai for Cell line studies.

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Cite this article as:

M. Nanthini *et al*. Cytotoxic activity of probiotic Bacillus subtilis SK09 against human colon cancer cell line (HT-29). Int. Res. J. Pharm. 2017;8(4):105-109 http://dx.doi.org/ 10.7897/2230-8407.080458

Source of support: Nil, Conflict of interest: None Declared