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. In recent years, great progress has been made in cancer detection and treatment. Cancer detection is a very crucial step in the diagnosis of cancer. The comparatively large number of polyps can be eliminated by performing screening and detection in the previous stage of colorectal cancer. Colon cancer risks can be prevented by probiotics as specified by the in vitro and in vivo studies. Reduction of H2O2 levels that lead to tumour progression can be controlled by introducing an effective strain namely Lactococcus lactis, which is investigated in DMH induced murine model.

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 and also can survive at the internal gut temperature by expressing its probiotic activity. 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 15 psi. 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 at 150rpm.
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 280°C at 250°C. The active principle isolation was carried out by Product extraction.

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 CO₂ at 37°C.

In vitro assay for anticancer activity (MTT assay)

In 24-well plate, the HT-29 cells of concentration 1 × 10⁵/well were plated and were incubated at 37°C with 5% CO₂. Once the cells reach the required confluence, the different concentrations of the sample were added and were incubated at 37°C with 5% CO₂ 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 4-(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>
<thead>
<tr>
<th>Compound name</th>
<th>Retention time</th>
<th>Mol. Formula/ Weight</th>
<th>Peak area (%)</th>
<th>Bioactivity/References</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-Octadecenoic acid, methyl ester</td>
<td>18.9</td>
<td>C₁₈H₃₆O₂ 296.4879</td>
<td>35.51</td>
<td>Antimicrobial [19] and anti-carcinogenic activity [20]</td>
</tr>
<tr>
<td>2,7-Diphenyl-1-6 dioxopyridazino (4,5:2’3’) Pyrrolo (4’,5’- d) pyridazine</td>
<td>22.97</td>
<td>C₂₇H₁₈N₂O₂355.34952</td>
<td>15.79</td>
<td>Cytotoxic against human cancer cell line [21]</td>
</tr>
<tr>
<td>Octadec-9-enoic acid</td>
<td>19.75</td>
<td>C₁₈H₃₆O₂ 282.46</td>
<td>18.46</td>
<td>Anticancer &amp; antileukemic activity [19, 22-24]</td>
</tr>
<tr>
<td>Hexadecanoic acid, methyl ester</td>
<td>17.18</td>
<td>C₁₈H₃₆O₂ 270</td>
<td>20.25</td>
<td>Antimicrobial [25] and anticancer [26] activity</td>
</tr>
<tr>
<td>4-Piperidineacetic acid ,1-acetyl 5- ethyl-2- (3(2-hydroxyethyl)-1H-indol-2-yl)-a- methyl, methyl ester</td>
<td>22.97</td>
<td>C₁₅H₂₀N₂O₂ 234.29422</td>
<td>25.13</td>
<td>Antimicrobial activists [27] and Anticancer activity [28]</td>
</tr>
</tbody>
</table>
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 dioxopyrazinono (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-Piperidinacetic 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
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-1-6-dioxopyridazino (4, 5:2’3’) pyrrolo(4’, 5’-d)pyridazine;
(iii) Octadec-9-enoic acid;
(iv) Hexadecanoic acid, methyl ester;
(v) 4-Piperidineacetiacid,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-Piperidineacetiacid, 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.

ACKNOWLEDGEMENTS

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


Cite this article as:

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

Disclaimer: IRIJ is solely owned by Moksha Publishing House - A non-profit publishing house, dedicated to publish quality research, while every effort has been taken to verify the accuracy of the content published in our journal. IRIJ cannot accept any responsibility or liability for the site content and articles published. The views expressed in articles by our contributing authors are not necessarily those of IRIJ editor or editorial board members.