Chapter - 4

Cytotoxicity and anticancer studies of Bacillus cereus and Bacillus pumilus metabolites
4.1. Introduction

4.1.1. Cancer: A major global health problem

Cancer is characterized by uncontrolled and invasive growth of cells. These cells may spread to other parts of the body and this is called metastasis. There are more than 100 types of cancer characterized by abnormal cell growth. There are many different causes ranging from radiation to chemicals to viruses; an individual has varying degrees of control over exposure to cancer-causing agents. Cancer still remains one of the most serious human health problems. Each year tens of millions of people are diagnosed worldwide with cancer and more than half of these patients eventually die from this disease. In 2002, 10.9 million new cancer cases (excluding skin cancer) were diagnosed and the number of deaths caused by this disease reached 6.7 million (Hayat, 2008; Jemal et al., 2011). Based on global cancer statistics, 12.7 million cases of cancer were detected in 2012 and cancer is a leading cause of death accounting for 8.2 million deaths in 2012 worldwide (Ferlay et al., 2013). In the industrialized western countries cancer accounts for about one fifth of all deaths. Likewise, one person out of three will be treated for a severe cancer in their lifetime. In Europe, cancer has become a major public health problem with an estimated prevalence of about 3%, increasing to 15% at old age (Parkin et al., 2005). This rising burden demands a clear and coordinated response from oncologists, public health professionals, policy-makers and researchers.

4.1.2. Microorganisms and cancer

Many microorganisms are known to cause cancer. Some examples are *Helicobacter pylori* which causes gastric cancer in humans and animals (Cover et al., 1995), *Salmonella typhi* associated with gallbladder cancer (Mager, 2006), *Streptococcus bovis* associated with colorectal cancer (Gold et al., 2004) *Chlamydia*
pneumoniae is associated with lung cancer (Kocazeybek, 2003) and Agrobacterium tumefaciens causes crown gall in plants (Wood et al., 2001).

4.1.3. Conventional cancer treatments

Conventional anticancer therapies consisting of surgical resection, radiotherapy and chemotherapy are effective in the management of many patients. But for about half of cancer sufferers these are ineffective. They often fail to achieve a complete cancer remission. Moreover, it has been widely recognized that radiotherapy and/or chemotherapy are likely to cause significant side effects. This has prompted the development of many new approaches for the treatment of cancer. One such example involves the use of live attenuated bacteria or their purified product (Bernardes et al., 2010; Patyar et al., 2010).

4.1.4. History of microbial-based cancer therapy

The use of bacteria in the regression of certain forms of cancer has been recognized for more than a century (Chakrabarty, 2003). The observation that bacteria could be used as anticancer agents dates back 150 years. The German physicians W. Busch and F. Fehleisen separately observed that certain types of cancers regressed following accidental erysipelas (Streptococcus pyogenes) infections that occurred whilst patients were hospitalized. In 1890, William B. Coley, a surgeon in the Memorial Hospital in New York, described for the first time bacteria as anticancer agents. He noticed that one of his patients suffering from neck cancer began to recover following an infection with erysipelas. He later observed that many of his patients with various forms of cancer had their tumors regress when they were infected with bacterial pathogens. Treatment to eliminate the infections allowed the cancer to come back (Nauts et al., 1946; Coley, 1991). Coley was so
excited with this finding that he devoted his career to researching the use of bacteria for cancer therapy. In 1935, Connell used sterile filtrates from *Clostridium histolyticum* to treat advanced cancers and observed tumor regression, which he pointed to be the result of enzymes production. In 1947 it was shown that the injection of spores of *Clostridium histolyticus* caused oncolysis then referred to as “liquefaction,” of a transplanted mouse sarcoma (Wei *et al.*, 2008).

Many scientists followed Coley with initial excitement of finding new microbes as anticancer agents. None succeeded in finding a cure for cancer due to the toxicity complications arising from these types of therapies. Several decades after Coley’s work, interest re-emerged in the use of bacteria to treat solid tumors. Experiments showed that pathogenic species of the anaerobic Clostridia were able to proliferate preferentially within the necrotic (anaerobic) regions of tumors in animals compared to normal tissues. This resulted in tumor regression but was accompanied by acute toxicity and most animals became ill or died.

### 4.1.5. Microbial-based cancer therapy

Microbial-based therapy of cancer is one of the emerging cancer treatment modalities. Important advances have been made to study and develop live bacteria or bacterial products such as proteins, enzymes, immunotoxins and secondary metabolites of bacteria and fungi which specifically target cancer cells and cause tumor regression through growth inhibition, cell cycle arrests or apoptosis induction (Frankel *et al.*, 2000; Jayadev *et al.*, 2004; Lam., 2007; Wei *et al.*, 2008; Bernardes *et al.*, 2010).
4.1.6. Bacteria in cancer therapy

Researchers have screened a number of anaerobic bacterial species like *Bifidobacteria*, *Lactobacilli* and pathogenic *Clostridia* for their ability to regress tumors in experimental animals. *Clostridium novyi* was found to be the most successful candidate to demonstrate significant anti-tumor effects. Facultative anaerobic bacteria such as *Salmonella* are also known to target tumor cells for growth and proliferation (Gill *et al*., 1996). Similarly, the use of *Shigella* (Sizemore, *et al*., 1995) and *Clostridia* (Fox *et al*., 1996) species targeting tumor cells has been reported. With growing research in this field new strains of bacteria are being investigated as anticancer agents like *Salmonella choleraesuis*, *Vibrio cholerae*, *Listeria monocytogenes* and even *Escherichia coli* have all been shown to replicate within tumors (Springer *et al*., 2005).

4.1.7. Bacterial vaccines and cancer

Many bacteria have been used to reduce the growth rate or size of tumors. The most prominent example is the use of *Mycobacterium bovis* (BCG), the vaccine strain, in the treatment of bladder cancer. Several studies have shown a relationship between the use of *M. bovis* BCG immunophrophylaxis after surgical removal of the tumor and the decreased recurrence rate or the delayed period in recurrence of tumors (Lamm *et al*., 1980; Matsumoto *et al*., 2002). The research into its mechanism of action revealed that it was capable of stimulating an immune response to attack and destroy the tumors.

Similar to *M. bovis* BCG which has been used to bolster the immune system against specific cancers such as bladder cancer, attenuated bacterial vaccine vectors such as *Listeria monocytogenes* and *Salmonella enterica* serovar *Typhimurium* were
also tested. These organisms target the antigen-presenting cells and also powerful inducers of an innate immune response and immune mediators such as IL-12. Hence, they have been recommended for use in cancer prevention and therapy (Pan et al., 1995; Paglia and Guzman, 1998).

**4.1.8. Oncolytic viruses in cancer treatment**

Oncolytic viruses can cause lysis of cancer cells. Once the cancer cells are ruptured, they are processed by the adaptive immune system, which can then target similar cells in other parts of the body. Many viruses, such as vaccinia virus, newcastle disease virus, reovirus and adenovirus are investigated to modify to achieve selective replication and killing of tumor cells. But the effective use of such viruses is sometimes hindered by the production of potentially neutralizing antibodies generated against them (Parato et al., 2005).

**4.1.9. Some anti-cancer microbial drugs**

Microbial drugs are making their unremitting influence as cancer chemotherapeutic agents. The discovery of actinomycin has led to venture into microbial world in the quest for anticancer compounds. Among the approved products deserving special attention are actinomycin D, anthracyclines, bleomycin, mitosanes, anthracenones (mithramycin, streptozotocin and pentostatin), enediynes (calcheamycin), taxol and epothilones. Actinomycin A, antibiotic isolated from *Streptomyces antibioticus* has served well against Wilms tumor in children (Sivaramkrishnan and Mahajan, 2009).

The anthracyclines are some of the most effective antitumor compounds developed and are effective against more types of cancer than any other class of chemotherapy agents. They are used to treat a wide range of cancers including
leukemia, lymphomas, breast, uterine, ovarian and lung cancers. Among the anthracyclines the first anthracycline discovered was daunorubicin (Daunomycin) in 1966, which is produced naturally by *Streptomyces peucetius*. Doxorubicin (Adriamycin) was developed in 1967. Another anthracycline “Epirubicin”, approved by USFDA in 1999 is favored over doxorubicin in some chemotherapy regimens as it appears to cause fewer side effects. Epirubicin is primarily used against breast and ovarian cancer, gastric cancer, lung cancer and lymphomas. Valrubicin is a semisynthetic analog of doxorubicin approved as a chemotherapeutic drug in 1999 and is used to treat bladder cancer. Bleomycin is a non-ribosomal glycopeptide produced by the bacterium *Streptomyces verticillus* and was first approved by USFDA in 1973 (Umezawa *et al.*, 1966). Mitosanes are excellent antitumor agents. Mitosanes (several mitomycins) are formed during the fermentation of *Streptomyces caespitosus*. They have limited utility owing to their toxicity. Mitomycin C, approved by the FDA in 1974 has shown activity against several types of cancer (lung, breast, bladder, anal, colorectal, head and neck) including melanomas and gastric or pancreatic neoplasms. Mithramycin (plicamycin) is an antitumor cum antibacterial aromatic polyketide produced by *Streptomyces argillaceous*. It is one of the drugs used in the treatment of testicular cancer, disseminated neoplasms and hypocalcaemia. Streptozotocin, a glucosamine-nitroso-urea, is a microbial metabolite with antitumour properties. It is produced by *Streptomyces achromogenes*. It is toxic to cells by causing damage to DNA, although other mechanisms may also contribute. The compound is selectively toxic to the β-cells of the pancreatic islets. In 1982, FDA granted approval for streptozotocin as a treatment for pancreatic islet cell cancer. Pentostatin, a purine analogue is an
anticancer drug produced by *Streptomyces antibioticus*. It interferes with the cell's ability to process DNA.

Calicheamicins are highly potent antitumor microbial metabolites produced by *Micromonospora echinospora*. Their antitumor activity is apparently due to the cleavage of double-stranded DNA. Taxol is reported to be produced by the endophytic fungi *Taxomyces andreanae* and *Nodulisporium sylviforme*. This compound inhibits rapidly dividing mammalian cancer cells. It was approved for refractory ovarian cancer. Today it is used against breast and advanced forms of Kaposi's sarcoma. The epothilones are macrolides originally isolated from a myxobacterium, *Sorangium cellulosum*. Moreover, they are generally 5 to 25 times more potent than taxol in inhibiting cell growth in cultures. Salinosporamide A, a novel anticancer compound from marine actinomycetes, *Salinispora tropica* is a potent proteasome inhibitor used as an anticancer agent and it had recently entered phase I human clinical trials for the treatment of multiple myeloma (Schein *et al.*, 1979; Minotti *et al.*, 2004; Mahajan *et al.*, 2007; Sivaramkrishnan and Mahajan, 2009).

All the above reports were the basis for the screening of our two bacterial isolates for their anticancer potential. The other reason for screening the metabolites for anticancer activity was due to their good antioxidant activity exhibited by ABTS method which proves that they have free radical scavenging activity (Borek *et al.*, 1997; Zimmermann *et al.*, 2001).

Hepatocellular carcinoma is one of the most common malignancies throughout the world (Johnson, 1996). It is characterized by its high incidence in hepatitis B virus-associated liver diseases (Graham *et al.*, 1996). The investigation
Evaluation of antimicrobial and pharmacological activities of microbes

of anticancer activity in the present work was targeted against hepatocellular cancer cells.

4.2. Review of Literature

Microorganisms are known to produce metabolites with anticancer properties. Literature survey was mainly concentrated on bacteria with anticancer properties. Literature survey was made on Bacillus species in particular because the metabolites of present isolates tested belongs to the same genera, i.e. Bacillus cereus and Bacillus pumilus. The survey included fungi with anticancer properties. The literature survey was also made on microbes (both bacteria and fungi) having antimicrobial activity with anticancer properties. This survey was made because many compounds which were first discovered as antibiotics failed in their later stages of development as antibiotics but proved to be good antitumor, antimigraine, immunosuppressive and antiparasitic agents (Demain, 1999). The two bacteria tested in the present study for anticancer properties have already proved to be good antibiotic producers.

4.2.1. Bacteria with anticancer activity

Malmgren and Flanigan (1955) reported treating of tumors with Clostridium tetani spores. The tumor-bearing mice died 48 hours late after the treatment in contrast to the healthy controls. In 1947, it was shown that the injection of spores of Clostridium histolyticus caused oncolysis then referred to as “liquefaction,” of a transplanted mouse sarcoma.

Carey et al. (1967) using Clostridium oncolyticum M-55 showed the benign activity of the strain by injecting themselves without any dangerous effect. It was tested against several transplanted tumors in different animal models.
A clinical trial using *Clostridium oncolyticum* M-55 spores to treat glioblastomas resulted in oncolysis with almost all glioblastomas being converted into brain abscesses one week after injection (Heppner and Mose, 1978).

Hanada *et al.* (1992) reported an unidentified *actinomycete* strain Q996-17 to produce a new compound epoxomicin. Epoxomicin exhibited strong *in vitro* cytotoxicities against various tumor cell lines whereas it did not exhibit antibacterial and anti-fungal activities. It showed strong *in vivo* inhibitory activity against B16 melanoma but moderate activity against P388 mouse leukemia.

Brandau *et al.* (2001) treated mononuclear cells with BCG for 7 days and demonstrated the ability of the BCG-activated killer cells to significantly destroy bladder tumor cells. Similarly, using C57BL/6 wild-type mice, NK deficient beige mice and mice treated with anti-NK1 monoclonal antibody, it was noticed that viable BCG cells significantly prolonged survival in wild-type mice compared with control non-treated mice. The BCG therapy was completely ineffective in NK-deficient beige mice or in mice treated with anti-NK1 monoclonal antibody.

IH-901, an intestinal bacterial metabolite derived from protopanaxadiol-type saponins of Panax ginseng has been reported to possess antitumor effects including inhibition of invasion, metastasis and angiogenesis and induction of tumor cell apoptosis. The effects of IH-901 on tumor promotion and related molecular events were examined in mouse skin *in vivo*. Mouse ear edema induced by the prototype tumor promoter 12-O tetradecanoylphorbol-13-acetate (TPA) was repressed by IH-901 pre-treatment in a dose-dependent manner (Lee *et al.*, 2005).

A new actinomycete strain, isolated from humus soils in the Western Ghats, was found to be an efficient pigment producer. The strain, designated AAA5, was
identified as *Streptomyces aurantiacus*. A yellow compound was derived from the extracted pigment and was identified as the quinone-related antibiotic resistomycin. It showed potent cytotoxic activity against cell lines viz. HepG2 (hepatic carcinoma) and HeLa (cervical carcinoma) *in vitro*, with growth inhibition (GI (50)) of 0.006 and 0.005 µg/ml, respectively (Vijayabharathi et al., 2010).

Shweta *et al.* (2013) has reported isolation of Camptothecine (CPT), a quinoline alkaloid known to be a potent inhibitor of eukaryotic topoisomerase-I. Because of this activity, several semi-synthetic derivatives of CPT are in clinical use against ovarian and lung cancers. They have reported the production of CPT by endophytic bacteria isolated from *Miquelia dentate* Bedd (Icacinaceae).

### 4.2.2. *Bacillus* species with anticancer activity

Jeong *et al.* (2008) isolated 93 marine bacteria from sea water samples. Of these, strain BIT-33 exhibited the strongest cytotoxic activity on three colon cancer cells (HT-29, SW480 and HCT116). Biochemical tests and 16S rDNA sequencing of this strain were carried to identify BIT 33 and the strain was identified as *Bacillus vallismortis*. The compound induced apoptosis of colon cancer cells, as indicated by DNA fragmentation of agarose gel electrophoresis, flow cytometric analysis and annexin V staining.

Ohba *et al.* (2009) reported “Parasporin” (PS), a collection of genealogically heterogeneous cry proteins synthesized in *Bacillus thuringiensis*. Parasporin proteins are found to have a strong cytocidal activity preferential for human cancer cells of various origins. At present, this protein group has been classified into four families: PS1, PS2, PS3 and PS4. There is strong evidence that PS1 kills cancer cells through apoptosis.
A novel marine microorganism with anticancer properties, H31, the metabolic product of *Bacillus* SW31, was isolated. The cell viability and apoptosis assays were performed. It had shown anti-tumor effects on head and neck cancer cells during MTT assay and potential for apoptotic-enhancing anticancer treatment of affected patients. The therapeutic effects of H31 were explored in mouse xenograft model and drug toxicity of H31 was examined in zebrafish model. Tumor growth in mice was suppressed by H31. In addition, in a zebrafish model used for toxicity testing, a considerable dose of H31 did not result in embryo or neurotoxicity (Lim *et al.*, 2010).

Li *et al.* (2012) isolated a marine-derived bacterium *Bacillus subtilis* strain B1779. The chemical investigation of culture broth of *B. subtilis* strain resulted in the isolation of 11 amicoumacins, including four novel lipoamicoumacins that were designated as lipoamicoumacins A–D (1–4), one new bacisarcin C (5) and six known analogues (6–11). Compounds 6 and 7 showed significant cytotoxic activity against HeLa cells.

### 4.2.3. Fungi with anticancer activity

An antitumor D-glucans from polypores was isolated called compound D-II from the culture mycelium of *Trametes versicolor*. The D-II strongly inhibited the growth of Sarcoma-180 transplanted subcutaneously in mice by intraperitoneal, intravenous, subcutaneous, and intramuscular administration at a dose of 5 mg/ml (Sugiura, 1980).

A cytotoxic monoterpene called montadial A was isolated from the polypore *Bondarzewia montana*. Montadial A was cytotoxic against lymphocytic leukemia
L1210 cells in mice at a concentration of 10 mg/ml as well as against promyelocytic human leukemia HL60 cells at 5 mg/ml (Sontag, 2004).

Klemke et al. (2004) reported isolation of compound called epiepoxydon from endophytic fungus *Apiospora montagnei* associated with the plant *Polysiphonia violacea* which exhibited significant cytotoxicity against human cancer cell lines.

Puri et al. (2006) reported isolation of compounds called Podophyllotoxin and aryl tetralin lignans from endophytic fungus *Trametes hirsute* associated with the plant *Podophyllum hexandrum* which exhibited anticancer activity.

Cytostatin analog protein phosphatase 2A (PP2A)-specific inhibitors are promising candidates of a new type of anticancer drug. To find new classes of PP2A-specific inhibitors, microbial metabolites were screened and found that rubratoxin A, a classical mycotoxin, was highly specific and potent inhibitor of the enzyme. While rubratoxin A inhibits PP2A, it hardly inhibited any other phosphatases examined. Rubratoxin A showed higher suppression of tumor metastasis and reduction of the primary tumor volume than cytostatin in mouse experiments. Rubratoxin A could be a good successor of cytostatin analogs leading to the development of antitumor drugs targeting PP2A (Wada et al., 2009).

Two new soil fungal isolates, *Emericella nidulans* and *Fusarium solani* were identified, characterized and tested for anticancer properties isolated from Wady El-Natron in Egypt. The metabolites of both isolates were tested against colon cancer Caco-2 (ATCC) cell line. Transcription-PCR was carried out to detect level of expression of p53 in Caco-2 cell line. Treatment with the two isolates caused p53
expression in Caco-2 cell line. The results of the two isolates were promising to be used as anticancer compounds (Mohamed, 2012).

4.2.4. **Microbial metabolites with antimicrobial and anticancer properties**

Aqabamycin A, B, C, and D belonging to the class nitro maleimide and aqabamycin E, F and G belonging to the class Maleimide oxime was reported from *Vibrio* sp. having anticancer properties. All these compounds were originally isolated with antibacterial properties (Yao et al., 2010). Pelagiomicin C belonging to the class Phenazine produced by *Vibrio* sp. and Anguibactin belonging to the class Catechol hydroxamate were reported from *V. anguillarum* with anticancer properties (Jalal et al., 1989; Imamura, 1994; Sato, 1995).

A novel antibacterial metabolite, ARK42 was isolated from a fungal strain *Aspergillus repens*. The metabolite exhibited antibacterial activity on human pathogens and also anticancer activity. Anticancer activity was demonstrated against human colon cancer DLD-1 and lung cancer LXFL529 cells (Park et al., 2002).

Brady et al. (2000) reported isolation of compound cytoskyrins from endophytic fungus *Curvularia lunata* associated with the plant *Niphates olemda* which exhibited antibacterial activity and potential anticancer properties.

Song et al. (2004) reported isolation of compound Aurasperone A and Rubrofusarin B from endophytic fungus *Aspergillus niger* IBF-E003 associated with the plant *Cynodon dactylon* which exhibited strong coinhibitors on xantin oxidase, colon cancer cell and some microbial pathogens.

Weber et al. (2004a) reported isolation of compound Brefeldine A from endophytic bacteria *Phoma medicaginis* associated with the plant *Medicago sativa*.
and *Medicago lupulina* which exhibited antibiotic activity and anticancer properties by initiation of apoptosis in cancer cells.

Organic extracts were obtained from bacteria-free algae and from surface-associated bacteria. Thirty-five bacterial strains were isolated from the surface of seaweeds and molecular identification by 16S rDNA sequencing showed they belong to the phyla Firmicutes, Proteobacteria and Actinobacteria. Pathogen strains were used to test antibacterial activity and HCT 116 colon cancer cells for anticancer activity. The strains Cc51 isolated from *Centroceras clavulatum*, Sm36 isolated from *Sargassum muticum*, and Eb46 isolated from *Endarachne binghamiae* showed anticancer activity, with IC50 values of 6.492, 5.531, and 2.843 μg/ml respectively (Luis et al., 2010).

Microbial extracts of 394 strains were evaluated on anti-proliferative activity against 4 cancer cell lines using MTT assay. Of these, 20 samples showed varying degree of cytotoxicity specifically on cancer cell lines and the growth of normal cells was not significantly affected by 1 mg/ml of each cell extracts. The 4 most potent extracts exhibiting growth inhibition on cancer cell type were selected for further studies. Cell morphological changes such as cell shrinkage, lose of surface contact and blebbing were observed in all treated cancer cells. DNA-binding dye staining demonstrated nuclear condensation and fragmentation. The cell lines used were cervical cancer (HeLa), liver cancer (HepG2) breast cancer (MCF-7), monocytic leukemia (U937) and African green monkey kidney (Vero) cell lines (Phonnok et al., 2010).

Maruna et al. (2010) isolated a new strain *Streptomyces* sp. AC113 an epiphytic bacteria occurring on the surface of roots of *Taxus baccata* L. The 16S
ribosomal DNA-based identification found that the new strain was 99% identical with *Streptomyces flavidofuscus*. The strain produced three major metabolites identified as methyltetrangomycin (1), methyltetrangulol (2) and hydroxytetrangomycin (3). Isolated angucycline metabolites showed significant antimicrobial activity against *Bacillus cereus* and *Listeria monocytogenes*. Cytotoxic activities of compounds 1, 2 and 3 against four cell lines (B16, HT-29 and non-tumor V79, L929) were evaluated. Compound 3 was the most potent anticancer agents with IC50 at 0.054 µg/ml against cell line B16.

In the process of screening the bacteria that can antagonize *Xanthomonas oryzae* (a pathogen of rice), a strain of *Bacillus subtilis* was isolated that could secret a novel antibiotic (named BS). BS had better in vitro anticancer activity. The IC50 for nine cancer cell lines was determined by MTT assay. In KM mice bearing sarcoma S180 that was treated with subcutaneous injection of antibiotic BS, marked cures were obtained (Xiaoxi, 2011).

Although, many literatures reported the screening of bacterial metabolites for anticancer properties, no literatures were available reporting the screening of *B. cereus* and *B. pumilus* metabolites for anticancer properties. This prompted to take up screening of *B. cereus* and *B. pumilus* metabolites for anticancer activities.

### 4.3. Materials and Methods

#### 4.3.1. Test samples

The samples prepared by successive solvent extraction with petroleum ether, ethyl acetate and further fractionation by dissolving in methanol, and methanol extract from the metabolites of *Bacillus cereus* (BC-1 to BC-5) and samples prepared similarly from *Bacillus pumilus* (BP-1 to BP-5) were used as test samples.
The entire above numbered sample fractions BC-1 to BC-5 and BP-1 to BP-5 were subjected to in vitro cytotoxicity studies by MTT assay.

4.3.2. In vitro cytotoxicity studies by MTT assay

4.3.2.1. Cell lines used

1) Chang liver cells (Normal human liver cell lines)

2) HepG2 (Human cancerous liver cell lines)

3) Hep2 (Human laryngeal epithelial carcinoma cell lines) (National Centre for Cell Sciences, (NCCS) Pune, India).

4.3.2.2. Growth medium

The cells lines were cultured in minimum essential medium (MEM). The medium also had 10% fetal calf serum, penicillin (100 U) and streptomycin (100 μg) (Sigma Chemicals Co., St. Lious, MO, USA).

4.3.2.3. Passaging the cells

All the reagents were brought to 37°C before use. Sufficient amount of trypsin-phosphate-versene-glucose (TPVG) (Merck Pvt. Ltd, Mumbai, India) solution was added to cover the monolayer, rinsed and discarded. Fresh TPVG solution was added and allowed to stand at room temperature for 2-3 minutes. TPVG solution was discarded and the flask containing the monolayer was incubated at 37°C for 3-5 minutes and slightly tapped to free the cells from the surface. 10 ml of MEM containing 10% fetal calf serum was added to the flask and pipetted to breakdown the clumps of cells. Total cell count was taken using a haemocytometer. The total numbers of cells were calculated. The medium was added according to the cell population needed. Required amount of medium containing the required number of cells (0.5-1.0x10^5 cells/ml) was transferred into bottles according to the
cell count. The volume was made up with medium and required amount of serum (10% growth medium and 2% maintenance medium) was added. The flasks were incubated at 37°C and the cells were periodically checked for any morphological changes and contamination. After the formation of monolayer, the cells were further utilized.

4.3.3. Cytotoxicity screening by determination of mitochondrial enzyme succinate dehydrogenase synthesis by Microculture Tetrazolium (MTT) assay

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4,5 dimethyl thiazole-2 yl)-2,5-diphenyl tetrazolium bromide (MTT) into a blue colored product (formazan) by mitochondrial enzyme succinate dehydrogenase on which the essay is based. The number of cells was found to be proportional to the extent of formazan production by the cells used.

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0x10^5 cells/ml using medium containing 10% new born calf serum. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once and 100 μl of different test samples was added to the cells in microtitre plates. The plates were incubated at 37°C for 3 days in 5% CO₂ atmosphere. Microscopic examination was made and observations were recorded every 24 hours. After 72 hours, the test sample solutions in the wells were discarded and 50 μl of MTT (Sigma Chemicals...
Co., St. Lious, MO, USA) in MEM (prepared in Hank’s Balanced Salt Solution (HBSS) without phenol red, 2mg/ml) was added to each well. The plates were gently shaken and incubated for 3 hours at 37°C in 5% CO₂ atmosphere. The supernatant was removed and 50 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm (ELISA Reader, Biotek) (Ribeiro et al., 2004). The percentage growth inhibition was calculated using the formula given below:

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\% \text{ growth inhibition} = 100 - \frac{\text{mean } OD_{540} \text{ of individual test group}}{\text{mean } OD_{540} \text{ of control group}} \times 100
\]

4.3.4. Nuclear staining studies

Induction of apoptosis is one of the important criteria for anticancer activity. Different morphological changes will occur during apoptosis like nuclear condensation, DNA fragmentation, membrane blebbing etc. In order to observe the alteration or morphological changes in the nucleus specific fluorescent dyes which will reemits visible light upon absorbing ultraviolet light are used. Acridine orange is a dye which stains nucleus as green and cytoplasm, red in color.

Nuclear staining studies were carried on HepG2 cell lines. The samples tested were BC-1 and BC-3. Single cover slip was placed in each well of 6 wells microtitre plate. The monolayer cell culture HepG2 cells (Human liver cancer cells) was trypsinized and cell count was adjusted to 50,000 cells/ml and 2.5 ml of cell suspension was added drop by drop on cover slip in each well. After 24 hrs or partial monolayer forms different concentrations of test sample BC-1 and BC-3 were prepared in maintenance medium were added. The control well received only maintenance medium. The plates were incubated at 37°C in 5% CO₂ atmosphere.
After overnight incubation, medium from wells were discarded and cells were washed with PBS. The cells were fixed with 1 ml of methanol (90%) at -20 °C for 20 minutes. The methanol was removed and replaced with acetone and kept for 10 sec. After fixing, cells were washed with ice cold phosphate buffer saline (PBS) 2-3 times. The cells were incubated with PBS containing 1% BSA and 0.1% triton X-100 at 37°C for 30 minutes. Plate was washed with PBS 2-3 times and 100 μl of Acridine orange (0.01% in PBS 7.4 pH) was added and incubated at 37°C for 20 minutes. The cover slip was washed thrice with PBS and placed on the slide. The cover slips were observed under fluorescent microscope for any nuclear changes and photographs were taken (Jayadev et al., 2004).

4.3.5. DNA fragmentation analysis

DNA fragmentation studies were carried on HepG2 cell lines. The samples tested were BC-1 and BC-3. In DNA fragmentation studies the DNA was extracted from the cancer cells. The extracted DNA was treated with the sample fractions BC-1 and BC-3. The control untreated DNA and DNA treated with the test samples BC-1 and BC-3 was subjected to agarose gel electrophoresis. The DNA fragmentation patterns were observed.

4.3.5.1. DNA extraction method

DNA was extracted from HepG2 cells (Human liver cancer cells). The liver cells were treated with digestion buffer (pH 7.5) containing 0.5% SDS, 25 mM tris-HCl, 0.5% mg/ml proteinase K and 5 mM EDTA at 55°C for one hour. After treating the cells with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1), DNA was precipitated with 3M sodium acetate (pH 5.2) and absolute ethanol, washed, dried and resuspended with tris-EDTA.
buffer containing RNase A (100 µg/ml) (Merck Pvt. Ltd, Mumbai, India) at 37°C overnight (Ribeiro et al., 2004).

4.3.5.2. Agarose gel electrophoresis

Agarose is a linear polymer extracted from a sea weeds. Purified agarose is a powder insoluble in water or buffer at room temperature but dissolves on boiling. Molten solution is then poured into a mould and allowed to solidify. As it cools, agarose undergoes polymerization i.e., sugar polymers cross-link with each other and cause the solution to gel. The density or pore size of which is determined by concentration of agarose.

DNA is negatively charged at neutral pH and when electric field is applied across the gel, DNA migrates towards the anode. Migration of DNA through the gel is dependent upon:

1. Molecular size of DNA
2. Agarose concentration
3. Conformation of DNA
4. Applied current

Matrix of agarose gel acts as a molecular sieve through which DNA fragments move on application of electric current. Higher concentration of agarose gives firmer gels, i.e., spaces between cross-linked molecules is less and hence smaller DNA fragments easily crawl through these spaces. As the length of the DNA increases, it becomes harder for the DNA to pass through the spaces, while lower concentration of agarose helps in movements of larger DNA fragments as the spaces between the cross-linked molecules is more.

The progress of gel electrophoresis was monitored by observing the migration of a visible dye (tracking dye) through the gel. Two dyes namely xylene cyanol and bromophenol blue were used in the study. These dyes migrate at the
same speed as double stranded DNA of size 5000 bp and 300 bp respectively. These tracking dyes are negatively charged, low molecular weight compounds that are loaded along with each sample at the start of run. When the tracking dye reaches towards the anode, run is terminated.

4.3.5.3. Preparation of agarose and electrophoresis

1X TAE was prepared by diluting appropriate amount of 50X TAE buffer (for one experiment, approximately 200 ml) and make up volume was made by adding 4 ml of 50X TAE to 200 ml with distilled water. One gm agarose was weighed and added to 100 ml of 1X TAE. This gives 1% agarose gel. Agarose was boiled till it dissolves completely and a clear solution results. A pinch of ethidium bromide was added to it and mixed well. The agarose solution was poured in central part of tank when temperature reached approximately 60°C. Before pouring the gel the comb of electrophoresis was placed so that it was set 2 cm away from cathode. Air bubbles were avoided during and after pouring the gel. Thickness of the gel was maintained around 0.5 to 0.9 cm. The gel was kept undisturbed at room temperature for the agarose to solidify.

1X TAE buffer was poured into the gel tank till buffer level stands at 0.5 to 0.8 cm above the gel. The comb was lifted gently, ensuring that wells remained intact. Power cords were connected to the electrophoresis power supply. Samples were numbered according to the well in which they were loaded. 2.5 µl of gel loading buffer was added to 25 µl of sample and minimum of 10 µl samples were loaded in the respective wells. Voltage was set between 50-100V and power was switched on. When the dye bands reached ¾ th length of the gel, the running was stopped. The bands were observed in UV light and gel picture captured, saved and
analyzed using alpha imager software (USA). (Agarose gel electrophoresis was performed as per available standard protocol of GENEi teaching kit, Bangalore, India.)

4.4. Results

4.4.1. In vitro cytotoxicity studies

Among the studied samples, the test sample BC-1 showed CTC$_{50}$ (cytotoxicity 50%) value of 225.36 $\mu$g/ml against HepG2, 152.196 $\mu$g/ml against Hep2 and 659.27 $\mu$g/ml against Chang liver cells. The CTC$_{50}$ values obtained for BC-3 sample was 228.266 $\mu$g/ml against HepG2, 282.1675 $\mu$g/ml against Hep2 and 351.8288 $\mu$g/ml against Chang liver cells. This result demonstrates sample fraction BC-1 is very much specific towards cancer cells followed by sample fractions BC-3. Both the sample fractions are compounds obtained from \textit{Bacillus cereus}. The other test samples BC-2, BC-4, BC-5, BP-1, BP-2, BP-3, BP-4 and BP-5 showed cytotoxicity towards both normal as well as cancer cells, and were not specific against cancer cells. The CTC$_{50}$ obtained for various test samples are shown in table 4.1. The histogram showing comparative activity of various test samples on normal cell line and cancer cell lines are shown in figure 4.1.
<table>
<thead>
<tr>
<th>Test samples</th>
<th>CTC&lt;sub&gt;50&lt;/sub&gt; in μg/ml</th>
<th>HepG2</th>
<th>Chang liver</th>
<th>Hep2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC-1</td>
<td>225.3682</td>
<td>659.2788</td>
<td>152.1960</td>
<td></td>
</tr>
<tr>
<td>BC-2</td>
<td>218.5247</td>
<td>207.4922</td>
<td>1050.2200</td>
<td></td>
</tr>
<tr>
<td>BC-3</td>
<td>228.2660</td>
<td>351.8288</td>
<td>282.1675</td>
<td></td>
</tr>
<tr>
<td>BC-4</td>
<td>147.0108</td>
<td>104.7945</td>
<td>97.9237</td>
<td></td>
</tr>
<tr>
<td>BC-5</td>
<td>323.2635</td>
<td>223.4996</td>
<td>715.2632</td>
<td></td>
</tr>
<tr>
<td>BP-1</td>
<td>237.3999</td>
<td>52.6892</td>
<td>282.1675</td>
<td></td>
</tr>
<tr>
<td>BP-2</td>
<td>209.0996</td>
<td>169.9896</td>
<td>109.6509</td>
<td></td>
</tr>
<tr>
<td>BP-3</td>
<td>200.0738</td>
<td>257.9673</td>
<td>159.9467</td>
<td></td>
</tr>
<tr>
<td>BP-4</td>
<td>179.0205</td>
<td>103.0957</td>
<td>54.6410</td>
<td></td>
</tr>
<tr>
<td>BP-5</td>
<td>350.2558</td>
<td>253.5986</td>
<td>310.1575</td>
<td></td>
</tr>
</tbody>
</table>

**BC** — *Bacillus cereus*; BC-1; Petroleum ether extract dissolved in methanol (Supernatant). BC-2; Petroleum ether extract dissolved in methanol (Precipitate). BC-3; Ethylacetate extract dissolved in methanol (Supernatant). BC-4; Ethylacetate extract dissolved in methanol (Precipitate). BC-5; Methanol extract.

**BP** — *Bacillus pumilus*; BP-1; Petroleum ether extract dissolved in methanol (Supernatant). BP-2; Petroleum ether extract dissolved in methanol (Precipitate). BP-3; Ethylacetate extract dissolved in methanol (Supernatant). BP-4; Ethylacetate extract dissolved in methanol (Precipitate). BP-5; Methanol extract.

Hep G2- Human cancerous liver cell lines
Chang liver- normal human liver cell lines
Hep2- Human laryngeal epithelial carcinoma cell lines
Figure 4.1. Comparative cytotoxicity values of the test samples on different cell lines.

BC – *Bacillus cereus*; BC-1; Petroleum ether extract dissolved in methanol (Supernatant). BC-2; Petroleum ether extract dissolved in methanol (Precipitate). BC-3; Ethylacetate extract dissolved in methanol (Supernatant). BC-4; Ethylacetate extract dissolved in methanol (Precipitate). BC-5; Methanol extract.

BP – *Bacillus pumilus*; BP-1; Petroleum ether extract dissolved in methanol (Supernatant). BP-2; Petroleum ether extract dissolved in methanol (Precipitate). BP-3; Ethylacetate extract dissolved in methanol (Supernatant). BP-4; Ethylacetate extract dissolved in methanol (Precipitate). BP-5; Methanol extract.

### 4.4.2. Nuclear staining studies

From the results obtained by nuclear morphology studies, it was evident that test samples BC-1 and BC-3 showed nuclear morphological changes similar to that of apoptotic cell morphology in cancerous cell culture HepG2. In normal cell culture tested, there was no such nuclear morphological change. This *in vitro* study has proved the selective toxicity of sample fractions BC-1 and BC-3 against cancer cells.
Evaluation of antimicrobial and pharmacological activities of microbes

The nuclear morphological changes observed by treating the test samples on cancer cell lines and normal cell lines are shown in figure 4.2.

![Figure 4.2](image)

**Figure 4.2.** Nuclear staining studies using acridine orange on normal HepG2 cells and BC-1 and BC-3 treated HepG2 cells

3a- Appearance of Normal HepG2 cells under fluorescent microscope
3b- Appearance of HepG2 cells treated with test sample BC-1 (200 μg/ml).
3c- Appearance of HepG2 cells treated with test sample BC-3 (200 μg/ml).
Arrows indicate membrane blebbing.

### 4.4.3. DNA fragmentation analysis

In DNA fragmentation analysis the control DNA showed intact band. The test samples BC-1 and BC-3 treated DNA showed damaged fragmentation patterns. The damaged DNA fragmentation patterns observed by treating the test samples on HepG2 cells are shown in figure 4.3.
**Lane 1:** Control untreated HepG2 cells

**Lane 2:** HepG2 cells + Test Sample BC-1 treated (200 µg/ml)

**Lane 3:** HepG2 cells + Test Sample BC-3 treated (200 µg/ml)

**Figure 4.3. DNA fragmentation studies of test samples BC-1 and BC-3**

**Lane 1:** Control untreated HepG2 cells single intact band was observed. This confirms that the DNA was intact in control untreated cells.

**Lane 2:** HepG2 cells + Test sample BC-1 treated cells (overnight treatment): DNA was found to be damaged with fragmentation pattern. No intact band, smear of DNA was observed.

**Lane 3:** HepG2 cells + Test sample BC-3 treated cells (overnight treatment): DNA was found to be damaged with fragmentation pattern. No intact band, fragmented DNA is seen.

**4.5. Discussion**

Many live bacteria such as *Clostridium, Bifidobacterium, Salmonella, Mycobacterium, Bacillus* and *Listeria* have been reported with the ability to selectively target cancer cells by grooving in the hypoxic core regions of solid tumors (Fialho et al., 2008). Not only live bacteria but also bacterially-derived products have been tested and some of them are successfully used. The mode of action is through the production of cytotoxic factors, enzymes, antibiotics and other secondary metabolites. SSL proteins produced by *Staphylococcus aureus* capable
Evaluation of antimicrobial and pharmacological activities of microbes

of binding “G-protein” receptors which are over expressed in cancer cells, 
*Mycoplasma arginini*, producer of an enzyme called “Ma-ADI”, inhibiting tumor 
growth and “Epothilones”, a cytotoxic metabolite with anticancer activity secreted 
by *sporangium cellulosum* are few examples (Barile et al., 1968; Fumoleau et al., 
2007; Walenkamp et al., 2009).

The two bacterial isolates tested for anticancer activity in the present work 
belongs to the genera *Bacillus*. The literature survey suggests that many *Bacillus* 
species have shown anticancer activities. A marine *Bacillus* SW31 has shown 
growth inhibition and apoptosis with head and neck cancer cells (Lim et al., 2010). 
Li et al. (2012) have reported *Bacillus subtilis* strain B1779 producing four novel 
lipoamicoacylins (1-4) and six known analogues (6-11). Among these compounds 
6–7 showed significant cytotoxic activity against HeLa cells. Xiaoxi, (2011) has also 
reported a strain of *Bacillus sublilis* that could secret a novel antibiotic (named BS). 
BS had better *in vitro* anticancer activity against 9 cancer cell lines when determined 
by MTT assays. The *in vivo* test In KM mice bearing sarcoma S180, when treated 
with subcutaneous injection of antibiotic BS, marked cures were observed. Jeong et 
al. (2008) have reported a compound from *Bacillus vallismortis* showing direct 
cytotoxic and apoptotic effect on colon cancer cells. Obha et al. (2009) have 
reported *Bacillus thuringiensis* strain with potential cytotoxic and anticancer activity 
against a number of cancer cell lines.

In the present study the two bacteria *Bacillus cereus* and *Bacillus pumilus* 
metabolites were tested for anticancer activity. The cytotoxicity results demonstrated 
the test sample BC-1 is very much specific towards cancer cells followed by test
sample BC-3. Hence, BC-1 and BC-3 test samples were taken up for further anticancer studies by nuclear staining method.

During nuclear morphology studies, it was evident that test samples BC-1 and BC-3 showed nuclear morphological changes similar to that of apoptotic cell morphology in cancerous cell culture HepG2. This in vitro study has proved the selective toxicity of sample fractions BC-1 and BC-3 against cancer cells. The promising results of nuclear staining was the basis for further screening of sample fractions BC-1 and BC-3 by DNA fragmentation analysis.

During DNA fragmentation studies carried on HepG2 cells the test samples BC-1 and BC-3 treated DNA showed damaged fragmentation patterns whereas the control DNA showed intact band which confirmed the anticancer properties of BC-1 and BC-3 test samples. Both test samples are metabolites obtained from Bacillus cereus.

In the present study the two bacteria that are tested for anticancer properties i.e. B. cereus and B. pumilus were originally isolated for their potential antibiotic production. Some of the important antitumor compounds used for chemotherapy are also antibiotics produced by bacteria (Sato, 1995; Imamura, 1997; Luis et al., 2010). Among the two bacteria tested only B. cereus exhibited anticancer properties. In the past B. cereus has been reported to produce enterotoxins (Thompson et al., 1984), antibacterial (Naclerio et al., 1993) and antifungal compounds (Ladeuze, et al., 2011) but not anticancer compounds. Jeong et al. (2008) have reported a compound from Bacillus vallismortis showing direct cytotoxic and apoptotic effect on colon cancer cells. In the present work the metabolites of B. cereus strain has also
demonstrated cytotoxic and apoptotic effect. The cancer cell lines used in the present work are HepG2 and Hep2 which are different from jeong et al work.

*Bacillus thuringiensis*, one of the closely related species to *Bacillus cereus*, has been reported to produce unique proteinaceous crystalline “parasporins” during sporulation with potential cytotoxic and anticancer activity against a number of cancer cell lines (Ohba *et al.*, 2009). The production of crystalline parasporins is the only character that discriminates between the two taxonomically closely related species, *Bacillus thuringiensis* from *B. cereus* (Logan, 2005). This report suggests that the compounds responsible for anticancer property of the present isolate *B. cereus* are different from parasporins produced by *Bacillus thuringiensis*. The mass spectral studies of TLC fractions of BC-1 and BC-3 has also showed that the compound is not a protein but of smaller molecular weight.

### 4.6. Conclusion

Cytotoxicity studies have confirmed that sample fraction BC-1 and BC-3 are toxic towards cancer cells. Nuclear staining studies have showed that sample fractions induce apoptosis cell death in liver cancer cells. DNA fragmentation studies have further confirmed that sample fractions cause damage to the cancer cell DNA. The mass spectral studies of the TLC fractions indicated that they are a group of compounds and are not proteins. Further purification of the TLC fractions and their spectral analysis may provide a lead molecule which can be taken up for *in vivo* activity and pre-clinical studies.