Discussion
5. Discussion:

The SML is known to influence exchange processes between the atmosphere and water bodies by physicochemical processes, e.g., the dampening of capillary waves induced by surface-active substances (Frew, 1997). Throughout the study period, both the temperature and salinity was fluctuated and recorded high in May and low at January. The salinity of the sea surface waters depends not only on the origin of the water mass but also on the evaporation from the surface (Kumaraguru et al., 2008). It was observed in the present study that the temperature and salinity were directly proportional. Earlier reports on the role of these parameters on the microbiology of SML is very limited whereas, in related studies it was reported that large number of bacteria are found at surface microlayers of both saline and freshwaters (Zavarzin, 1955; Dratchev et al., 1957; Bogorov, 1966; Harvey, 1966; Babenzien & Schwartz, 1970; Morita and Burton, 1970; Sieburth, 1971; Tsyban, 1971; Hatcher & Parker, 1974; Sieburth et al., 1976; Dutka & Kwan, 1978; Crawford et al., 1982; Danos et al., 1983; Münster et al., 1998). This indicates that salinity has no impact over the bacterioneuston distribution.

Ramos (2009) had studied the effect of physico chemical parameters on bacterioneuston and found that the bacterial abundance decreased from day to night. The probable reason ascribed for this decrease was not only the sun exposure but also other parameters like temperature and salinity. It was also noted that the bacterial abundance within the
underlying water increased from day to night. However, the differences were not statistically significant (p=0.423). Bacteria from the SML possibly migrate during the night towards the underlying water (Ramos, 2009). Hermansson & Dahlbaeck (1983) reported the migration of active bacteria from the SML to underlying waters.

Bacterioneuston may play a pivotal role in controlling physicochemical processes. However, determining whether the bacterioneuston is ‘successful’ remains controversial, with previous studies reporting conflicting results regarding bacterioneuston activity, abundance, and diversity compared to bacterioplankton (Maki, 1993).

5.1 Sampling devices

One of the major challenges in studying SML is the choice of sampling device. The thickness of the sampled SML and the mode of operation of the chosen sampling device, especially its degree of selectivity are the important factor to be considered. Earlier studies have examined the potential bias of SML sampling devices with respect to biological parameters (Hatcher and Parker, 1974; Agogue et al., 2004; Franklin et al., 2005). One such study has extensively examined the possible bias of Glass plate sampler (Agogue et al., 2004). The present study has revealed that Firmicutes was predominant and only eight strains belong to Gram negative. The sampler’s glass plate and teflon plate yield only gram positive bacteria. This may be due to the presence of inhibitory substance originating from the sampling devices themselves. Most authors of previous research
either did not mention how or if their sampling devices were treated, or reported treatment of the glass plate with Milli-Q water and ethanol (Agogue et al., 2004). In the present study, the glass plate and teflon plates were wiped with ethanol before sampling and still both the samplers yield a particular bacterial family.

Sampling devices differ in their mode of operation and in the material of the sampler itself (Huehnerfuss, 1981a & b). Based on the results of the present study, it is revealed that the glass plate and teflon plate has sampled SML more selectively without any contamination from underlying waters. This is evident from the fact that the SML isolates were distinct from the subsurface isolates with the dominance of phylum *Firmicutes* bacteria.

Franklin *et al.* (2005) used 47-mm diameter and 2-μm pore sized polycarbonate membrane to sample SML. The membrane was placed on the sea surface and attached to the surface by surface tension forces. Sub-surface water samples were also collected using sterile polycarbonate bottles. The results revealed that the bacterioneuston was distinctly different compared to the isolates of subsurface waters collected at 0.4 m. This membrane method which is said to be meant for isolation of bacterioneuston was also devised in the present study.
The membrane employed in our study was Polyethosulfonate (PES) membrane which is usually used in the water filtration system to filter the microorganisms from water. It provides removal of fine particles, bacteria, viruses, and fungi, making it a versatile membrane and is an inherently hydrophilic membrane that wets out quickly from the SML. The hydrophilic nature of PES means no added surfactants are used to increase wettability and is also extremely low protein binding so that this membrane has the potency to stick the microbes to the surface and hence PES membrane was used in our study. The biochemical results showed that membrane method yield some gram negative bacteria too besides Firmicutes. The weakness of this method is that it is difficult to avoid distortions of the sea surface microlayer from the boat due to the proximity of the sampling area to the boat hull. Kurata (2012) and Kurata et al. (2013) improved this method by attaching the polycarbonate membrane filter to a fishing line and by using the fishing rod to deploy this filter away from the boat and then bringing it to the boat. As the membrane is placed over sea surface, it is directly exposed to atmosphere and hence the possibility of contamination by air microbes is prominent.

Ultimately, we developed a sampling device (MMM) in which the membrane is not exposed to atmosphere. Unlike membrane method, in MMM, the membrane would not be placed on the sea surface, it is attached to the fabricated device and hence when the device is brought in close proximity to the SML due to surface tension and volatilization process the SML layer alone is effectively sampled. The air-sea interface is a unique
habitat characterized by high surface tension, high solar radiation, variable temperature, salinity and turbulence. Wallace and Duce, (1978) estimated that bubbles from SML could transport $4 \times 10^2$ pug Cm$^{-2}$s$^{-1}$ to the surface of open ocean waters. The samplers, membrane and MMM obtained eight gram negative strains which exhibit more or less similar biochemical pattern so that only one strain was subjected to 16s rRNA sequencing which revealed that it belongs to *P. plecoglossicida*. Apart from these, no other microbes were obtained from our sampling. This is contrary to the report that bacteria may be $10^2$–$10^5$ times more abundant in the SML than in subsurface waters (Bezdek & Carlucci, 1972; Sieburth *et al*., 1976) whereas, Bell and Albright (1982) has reported low microbial abundance and Agogué *et al*., (2005) reported no significant difference between SML and underlying water microbial abundance.

Zdanowski & Figueiras (1999) have presumed that the rich organic matter of SML supports the bacterial communities. The electrostatic interactions between viable bacteria and rising particles by a bubble flotation also increase the abundance of bacteria in the SML. This happens because living bacteria have a negative charge that, attending to the higher seawater pH, results in a passive passage by attracting cations (Grasland *et al*., 2003). The variability of microbial abundances reported in the literature might be related to the use of different sampling devices to collect the SML and/or to a natural ecological variety of the nutrient enrichment (Carlson, 1982a & b; Hardy, 1982; Agogué *et al*., 2004).
5.2 Phylogenetic analysis

To explore whether the bacterial communities inhabiting both the surface microlayer and underlying waters were consistently different, 43 bacterial isolates from SML (n=37) and underlying waters (n=6) were collected from the sampling locations and biochemically characterized. The isolates exhibiting different biochemical characters were selected for 16s rRNA sequencing studies. About 12 isolates were considered for the study. Out of 12 isolates, 11 were gram positive and only one gram negative was selected. As the morphology and gram staining of underlying water isolates clearly depict that they belong to *Staphylococcus sp.* and *Corynebacterium sp.*, only SML isolates were undergone for 16s rRNA sequencing studies. Briefly the DNA was obtained from the 12 isolates; 16s rRNA gene was amplified by using universal bacterial primers. From the BLAST search, all the gram positive isolates were found to be *Bacillus spp.* and the gram negative isolate was found to be *P. plecoglossicida*. The phylogenetic analysis revealed that 16s rRNA gene sequence similarities ranged from 97% to 100% to already known species. Particularly, around 25% of the isolated strains showed sequence similarities 100% to previously reported species, 67% of them have sequence similarities between 98-100% and 8% of them have sequence similarities lower than 98%. Such low similarity values (< 98%) indicate that we were able to bring into culture new marine genera or even new families (Devereux *et al.*, 1990). These observations agreed with other reports on the prevalence of novel marine bacteria among the culturable bacterial fraction (Pinhassi *et al.*, 1997; Hagström *et al.*, 2000; Bowman *et al.*, 1997; Suzuki *et
Agogue et al., 2005 reported the same and he concluded that although, they initially expected that these new culturable bacterial taxa might be the dominant bacterial components in the unexplored surface microlayer habitat, these potential “new” bacterial genera were not specific for the surface microlayer.

The phylogenetic relationship of *Bacillus spp.* indicated all of them exhibit different pattern. Even within *B. subtilis*, each one of them related to various bacteria. The isolates SML1, SML2, SML3, SML4, SML7 and SML9 were identified as *B. subtilis* from BLAST results. The taxonomic position of the bacterioneuston of this study was studied with the related marine and atmospheric bacteria. The isolates SML 1, 2, 3, 4, 7 and 9 (*B. subtilis*) exhibited a similar phylogenetic pattern which revealed that they are closely related to atmospheric bacteria. The branching pattern showed that SML isolates *B. subtilis* were closely related to *B. aerius*. This was supported by Norkrans (1980), who explained the dominance of *Bacillus sp.* in the surface microlayer by the fact that spore forming species coming from air are more resistant to desiccation and to solar radiation than the bubble transported marine species. The bacterioneuston isolates SML 5, 6, 11 and 12, the different species of *Bacillus* also exhibited the similar relationship with atmospheric bacteria except SML10 *B. anthracis* which indicated close relation with marine bacteria *B. litoralis*. Also the SML isolates showed a distant relation with deep sea bacteria which revealed that the sampling devices we used extricated the superficial
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SML which was between the atmosphere and subsurface water and hence the bacterioneuston obtained in our study showed relation with these zonal bacteria but doesn’t placed with the same nodal pattern of the tree. The unusual isolate *P. plecoglossicida* is a bacteria present in the fish *Plecoglossus altivelis* and the isolation of these bacteria from SML is surprising. In addition, it showed not much marine bacteria in its blast results except *P. xanthomarina* (from marine ascidian) and *P. proteolytica* (from Antarctica waters). It also showed sequence homology with bacteria from natural mineral waters such as *P. brenneri strain CFML 97-391* and *P. jessenii strain CIP 1052774* (Baida and Izard, 2001; Verhille *et al.*, 1999) and so SML isolate *P. plecoglossicida* might be transported to the sea *via* sea food industries or drinking water treatment plants which were operated on the Mandapam coast.

From the 16s rRNA sequencing results, it was observed that the *Firmicutes* bacteria were predominant from the SML of Palk bay (Mandapam) region. The presence of *γ*-proteobacteria was also observed. It was already reported that *Beta* and *ε*-proteobacteria and *Bacilli* were exclusively found on SML of Ria de Aveiro estuary in Portugal. It is also possible that despite an apparently larger diversity on SML, the predominance of one single class can be more evident in this layer (Ramos, 2009). In a study on Mediterranean sea SML by Agogue *et al* (2005), the bacterial community structure of SML was reported as *α*-proteobacteria, *γ*-proteobacteria and *Firmicutes*. In contrast, study of North Sea SML bacterial community structure revealed the presence of *Vibrio spp.* and *Pseudoalteromonas spp.* (Franklin *et al.*, 2005). In a comparison study of cultivable and
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non culturable methodology of SML bacteria, Azevedo et al (2012) isolated bacteria belonging to the genera *Pseudomonas, Aeromonas, Bacillus* and *Shewanella*. Hakvag et al (2009) isolated a new strain belonging to the genus *Collimonas* and *Streptomyces sp.* from the SML off the coast of Trøndelag, Norway.

The studies on bacterioneuston is limited and most of the studies manifested, that *Bacillus* genera and bacteria belonging to *Proteobacteria* family found in SML and also in some regions they are predominant. In the present study, the *Bacillus spp.* seems to be predominant in Palk bay (Mandapam) region and the presence of \(\gamma\)-*proteobacteria* was also observed. Whereas, Carty and Colwell (1975) reported in a bacterioneuston study of SML the *Bacillus* and *Micrococcus* species which were normally in the atmosphere. The influence of these terrestrial species cannot be excluded in coastal areas, but these species cannot be considered as neustonic bacteria.

From the present study, we hypothesize that most of the bacteria living in the sea surface microlayer may accumulate via a flotation process when they attach to particles and/or bubbles coming from the water column. A fraction of these bacteria may also accumulate *via* atmospheric deposition. As a consequence, viable cells collected from the surface microlayer may be those that have developed mechanisms to resist intense solar radiations and/or high concentrations of toxic compounds. We cannot disregard, however, the existence of resistance-forms in the surface microlayer, such as spores, that
could grow under laboratory conditions (Agogue et al., 2005). Hence, we can suggest that specific neustonic bacterial species are scarce rather than abundant. However, the existence of specific neustonic populations at the marine surface microlayer cannot be excluded, as some adapted species have already been described in freshwater environments (Glo¨ckner et al., 1998; Paddies et al., 2004). If this is the case for marine environments, they do not seem to be strongly enriched in the marine neuston of coastal areas.

5.3 Metabolites from bacterioneuston

To control the pathogenic diseases, researchers study the organisms of different environment and explore potential bioactive compounds for treatment. Thus began the chemistry of the natural products and biotechnology area for human welfare. Several of these organisms produce secondary metabolites, which are part of a wide variety of natural compounds used by humans to combat diseases. Secondary metabolites are defined as organic compounds formed as bio products in organisms, not directly related to growth, development and normal reproduction. Some examples are fibers (cotton, silk, wool); fuels (oil and natural gas), and medicines (antibiotics, hormones, vaccines).

The importance of finding and using these secondary metabolites can be justified in two ways (a) to know the natural substances that can be beneficial for man and (b) to identify the organisms that produce these substances in order to make a rational exploitation of them, because they may be the only carriers of useful compounds to combat pathogenic
microbes. Marine organisms are considered as one of the most inexhaustible source of useful chemical substances for the development of new drugs. The secondary metabolites of many marine algae, fungi, bacteria, sponges, etc. have been reported as bioactive compounds. Among the marine microorganisms, *Actinomycetes* are ‘high G+C content Gram-positive bacteria’ with an unparalleled ability to produce diverse secondary metabolites (Das *et al*., 2008). Of these *Actinomycetales* compounds, 75% were from *Streptomyces* and 25% were from rare actinomycetes (Berdy, 2005). The SML has gathered the attention of researchers in recent era and hence the details regarding its nature, nutrient composition, pollutants and microbiology were dealt. The study of secondary metabolites as bioactive compounds from bacterioneuston is very limited.

A new strain of bacterioneuston of the genus *Collimonas* that produce an antibacterial compound against *Micrococcus luteus* was reported by Hakvag *et al*., (2009). Subsequent studies using LC-MS identified this antibacterial compound as violacein, known to be produced by several marine-derived bacteria. The antibacterial activity of violacein against *E. coli* is reported to be low, even at high concentrations (de Azevedo *et al*., 2000; Yang *et al*., 2007).

In the present study, the crude extract of the *B. subtilis* was purified and the obtained elutions were tested for antibacterial activity against gram positive and gram negative human and fish pathogens. The fractions 3 and 5 showed promising results against human
Pathogens rather than fractions 2 and 6 but all the fractions have weak activity against fish pathogens. At different concentrations, the fractions 3 and 5 were tested against the pathogens. Even though, the fractions exhibit weak activity against fish pathogens, the activity was still observed at the lower concentration (5µg). Against Gram negative pathogens, the fraction 3 showed moderate activity and weak activity against gram positive pathogens at concentrations 15 and 20 µg. No zone of inhibition was seen at lower concentrations except *S. aureus*. On the other hand, fraction 5 showed good activity against gram negative pathogens and moderate activity against gram positive pathogens at concentrations 15 and 20 µg. Even in the lower concentrations, zone of inhibition was observed against all pathogens except *P. aeruginosa* and *V. cholerae*. This manifests that the fraction 5 has good antibacterial activity against both gram positive and gram negative pathogens. In contrast, fraction 3 has potential activity against gram negative pathogens only. As the fractions yield zone of inhibition against fish pathogens at lower concentrations, it can be considered as a potential metabolite to prevent fish spoilage in seafood processing industries.

Hakvag *et al.*, 2008 reported that *Actinomycetes* from SML exhibited potential antimicrobial activity. The extracts of the isolates were subjected to agar diffusion assays for antimicrobial activity against *Micrococcus luteus* ATCC 9341, *Candida albicans* ATCC 10231 and *Escherichia coli* K12. In this study, it was observed that 82% of the isolates showed antagonistic activity against at least one of the test pathogens. Several of
the isolates were active against more than one indicator organism. It was also reported that around 80% of the isolates acted against Gram-negative bacteria also inhibited Gram-positive bacteria, and *vice versa*. Hence, Hakvag *et al* concluded the fact that some *Actinomycetes* isolates displayed activity against more than one indicator organism may indicate production of several antimicrobial compounds and/or production of compounds with multiple microbial targets.

In a group of antibiotics, the inhibitory activity against Gram-positive and Gram-negative bacteria are 66% and 30% respectively but only 34% shows antifungal activity; for which 21% (3500) are active against yeasts, 11% (1800) against phytopathogenic fungi and 24% (4000) are active against other fungal species (Berdy, 2005). The antibiotics for gram positive bacterial and fungal pathogens are least when compared with gram negative bacteria. The metabolites of *B. subtilis* from SML showed potential antimicrobial activity against gram positive and gram negative bacteria as well as fungal pathogens.

The percentage of neuston actinomycete isolates displaying antimicrobial activity was found to be considerably higher than those reported previously. In the earlier studies, about 50% of isolated marine pelagic bacteria exhibited antagonistic properties against other pelagic bacteria (Long & Azam, 2001), and only 44% of *Streptomycetes* from the marine sediments have shown antibacterial activity (Peela *et al*., 2005). In the later study
by Hakvag et al., 2008, 17% of the isolates displayed antifungal activity. The lower degree of antifungal activity compared to antibacterial activity among Streptomyces species isolate from marine sediments was reported by Sponga et al., (1999). Selective response mechanisms exist against certain organisms, as shown in marine biofilms of Bacteriodetes, Planctomycetes, Proteobacteria, where the production of chemical substances such as violacein has been observed. This compound works as a defense mechanism against certain specific predators like the protozoa consuming bacteria. This allows the successful persistence of the bacterial biofilm in several marine environments (Matz et al., 2008). Thereafter, reports on neustonic bacterial metabolites were completely lacking.

The genus Streptomyces is well known for its antimicrobial activity irrespective of their habitat either terrestrial or marine. Hence, there is no surprise in the antimicrobial activity of Streptomyces from bacterioneuston. In our study, the metabolites of B. subtilis were tested for antibacterial activity. Studies were already reported that Bacillus species have been found to possess chemical compounds with anticancer activity. Although this Bacillus sp. can grow in almost any substrate, it is possible to suggest that this species seems to have acquired the mechanism to synthesize compounds capable of inhibiting HCT-116 colorectal cancer cells (Villarreal-Gómez et al., 2010). It is also observed that Bacillus sp. yield nearly 860 nos. of bioactive secondary metabolites and the reports suggest that they have potential anticancer activity (Berdy, 2005) and antibacterial
activity against gram positive pathogens (Ming and Epperson, 2002). It was also reported that some compounds such as polymyxin, colistin, and circulin exhibit activity against Gram-negative forms, whereas bacillomycin, mycobacillin, and fungistatin are effective agents against molds and yeasts (Katz and Demain, 1977). But in our present study, the metabolites of SML B. subtilis showed very good activity against gram negative bacteria too.

Many other related studies marked that, metabolically marine Bacillus strains are different from their terrestrial counterparts, and thereby, they may produce unique bioactive compounds, which are not found in their terrestrial counterparts (Jensen and Fenical, 1994; Feling et al., 2003). The ability to produce diverse classes of antibiotics by Bacillus spp. has been evidenced by several genomic studies. For example, the genome sequence of the widely distributed Bacillus strains revealed that about 8% of genome is devoted to synthesizing antibiotics (Chen et al., 2007; Kunst et al., 1997). Similarly, genome analysis of a marine B. subtilis subsp. spizzenii strain gtP20b, isolated from the Indian Ocean, indicated the presence of huge number of genes for biosynthesis of secondary metabolites (Fan et al., 2011). As our B. subtilis strain showed sequence similarity above 96% to B. subtilis subsp. spizzenii strain, it might also have the same genome sequences for antibiotic synthesis.
Many reports suggest a wide variety of structurally diverse antimicrobial compounds attributed to *Bacillus subtilis* (Stein, 2005), cyclic lipopeptides (LPs) of the surfactin, iturin and fengycin families have well-recognized potential for use in biotechnology and biopharmaceutical applications because of their surfactant properties (Ongenaa and Jacques, 2008). Iturins consist of a peptide ring of seven α-amino acid residues, with the constant chiral sequence LDDLLDL and common presence of D-Tyr2, closed by a β-amino fatty acid with 14 to 17 carbon atoms (Magnet-Dana and Peypoux, 1994). Although it has been shown that they exhibit a strong activity against pathogenic fungi and yeasts, their antibacterial activity has not been intensively investigated so far.

In the present study, antimicrobial bioactive compounds were isolated from the culture supernatant at the beginning of stationary phase. Beric *et al.*, (2012) reported that the antibacterial activity of *B. subtilis* was detected early in the logarithmic growth phase and reaching the maximum at the beginning of the stationary phase, with no significant decrease during prolonged incubation at 120 h. This indicates that sporulation had no effect on the production of antimicrobial substance, which was very stable in the culture even after several days of incubation. Apart from antibacterial and antifungal activity, there were some reports on *Bacillus sp.* in producing some biologically active compounds against cancer cell lines (Kim *et al.*, 2007; Ohba *et al.*, 2009).
5.4 Anticancer activity

The purified metabolites were *in vitro* tested for their cytotoxic potential on cancer cell lines such as Liver heptacellular carcinoma (HepG2) and Human breast adenocarcinoma (MCF-7). The ability to induce tumour cell apoptosis is an important property of a candidate anticancer drug (Frankfurt and Krishan, 2003). The basic cytotoxic assay to assess anticancer activity is MTT assay. In our study, the IC$_{50}$ values of MCF-7 were observed as 46.3µg/ml and 99.8µg/ml and for HepG2, the values were observed as 38.1µg/ml and 76.9µg/ml for the fractions 3 and 5 respectively. This indicated that fraction 3 showed intense activity against both the cell lines compared to fraction 5. Similar study on the cytotoxic study of HepG2 cells against a compound was studied by Marotta *et al.* (2007) revealed that the growth of HepG2 cells can be inhibited in concentration dependent manners and at 200 mg/L concentration the survival fraction decreased to nearly null. Besides the inhibitory effect of the compound on cell proliferation, high doses (100-200 mg/L) of compound significantly increased cytotoxicity as determined by LDH release in HepG2 cells. In our study, the highest dose used was 500µg/ml but even the low doses like 50 and 100µg/ml showed significant response.

Another cytotoxic study of MCF-7 against L-glutaminase and L-ascorbic acid by Nathiya *et al.*, (2012) showed that the IC$_{50}$ values were 98.53µg/ml and 80.52µg/ml respectively. The values were significantly higher when compared to the cytotoxicity of fraction 3.
Hence, compared to the previous studies noted above, the metabolites of our study showed potential activity against both the cancer cells. There may exist the possibility that apoptosis contributes the cytotoxic effect of the eluted fractions. The process of apoptosis is characterized by specific biochemical and morphological changes.

Apoptosis is an essential process for maintaining homeostasis in multi-cellular organisms and it is induced by receptor-regulatory signal such as hormones, cytokines and growth factors (Hayashi, 1972), thus playing crucial roles in a variety of physiological and pathological conditions. Many methods have been developed to test apoptosis including morphological study, biochemical assay and molecular studies of which morphological study is the most reliable method. The apoptosis processes of different cells in the same tissue are not synchronous. As reported by Catchpole and Stewart (1995), the appearance of DNA ladder is detected after the appearance of cell apoptosis by morphological observation. So the morphological observation, especially in the early stage of apoptosis, is very important for apoptosis test.

For the preliminary characterization of the cytotoxicity induced by the microbial extracts in the cancer cells, the changes in cell morphology induced by the treatment of purified fractions was first examined under a phase contrast microscope. In both the cancer cell lines, cell shrinkage, membrane blebbing and loss of cell adhesion were induced by the microbial metabolites. These cellular changes are the characteristics of the apoptotic
induction of cell death. To further characterize the cell death induced by the microbial extracts, nuclear morphology was determined by AO/EB staining in the presence and absence of purified fractions. Distinct apoptotic morphological changes including cell shrinkage, chromatin condensation and formation of apoptotic bodies were observed with AO/EB double staining by fluorescence microscope. Thus, it was suggested that the mode of cell death triggered by metabolites might be the process of apoptosis, which is recognized as a novel strategy for identification of anticancer drugs (Shougang et al., 2008; Hou et al., 2009). A similar study was done by Phonnok et al (2010) using cell lines such as HepG2, MCF-7, HeLa, U937, Vero cells and PBMC against the extracts of Bacillus sp and Pseudomonas sp. Hence, it is apparent that Bacillus sp. metabolites have potential anticancer property against cancerous cells but not against normal cells.

The activity of caspases which is known as an important biochemical feature in apoptotic signaling was further determined to investigate whether the apoptosis was induced by the purified fractions. Our results clearly demonstrated that the microbial metabolites induced apoptosis as detected from DNA fragmentation and caspase-3, 8 and 9 activation in MCF-7 and HepG2 cancer cells which seems to be an account for anti-proliferative activity as determined by the MTT assay. The cytotoxic responses of the cancer cells to the metabolites were dose dependent which was confirmed by the increased caspase expression at the rise of the concentration of purified fractions.
A similar study on caspase expression for anticancer activity was studied in the cell lines such as HepG2, MCF-7, HeLa, U937, Vero cells and PBMC against crude extracts of *Bacillus sp* and *Pseudomonas* (Phonnok *et al.*, 2010). The study showed that in normal cells, no anti-proliferative activity was observed even at 12-50 times concentration of microbial products. In addition, the microbial extracts specifically affected both slow- and fast-dividing cancer cells but not affected slow- and fast-growing normal cells; PBMC and Vero cells, respectively.

The fold expression of apoptosis regulator Bcl-2 and p53 was observed in this study. The Bcl-2 family is the best characterized protein family involved in the regulation of apoptotic cell death, consisting of anti-apoptotic and pro-apoptotic members. The anti-apoptotic members of this family, such as Bcl-2 and Bcl-xL, prevent apoptosis either by sequestering proforms of death-driving cysteine proteases called caspases (a complex called the apoptosome) or by preventing the release of mitochondrial apoptogenic factors such as cytochrome c and AIF (apoptosis-inducing factor) into the cytoplasm (Tsujimoto, 1998). Thus, the Bcl-2 family of proteins acts as a critical life–death decision point within the common pathway of apoptosis. The induction of anticancer agent would surpass the Bcl-2 and so the expression of Bcl-2 gene got reduced so that the apoptosis takes place.
Another apoptosis regulator studied in the present study is p53. The p53 gene is a tumor suppressor gene, i.e., its activity stops the formation of tumors. The p53 tumor suppressor acts to integrate multiple stress signals into a series of diverse anti proliferative responses. One of the most important p53 functions is its ability to activate apoptosis, and disruption of this process can promote tumor progression and chemoresistance. p53 apparently promotes apoptosis through transcription dependent and independent mechanisms that act in concert to ensure that the cell death program proceeds efficiently (Fridman and Lowe, 2003). As this is a tumour suppressor, the expression of p53 was increased due to apoptosis after the sample treatment.

A similar study about the expression of apoptosis regulator integrins using RT PCR in LN18 cells (Brain cancer) was studied after the addition of apoptosis inducing agents. In this study, β-actin was used as housekeeping control. The expression of β-actin and the integrin subunits increased by about a cycle indicating that the LN18 cells proceeded through apoptosis (Magro, 2013).

The present study showed increase in the fold expression of p53 and decrease in Bcl-2 after the addition of purified metabolites. The β-actin was used as housekeeping control. The cell line without the addition of metabolites was used as control for clearly understanding the expression of apoptosis regulator genes. The present study reported
that 10µg of fraction 3 and 50µg of fraction 5 manifested best results against MCF-7 and HepG2 cell lines.

Not only the specific cytotoxic effect to the cancer cells that made the metabolites a good candidate to develop as antitumor agent, but also the preliminary demonstration of apoptotic induction in cancer cells. Thus, to induce apoptosis in cancer cells is an important feature that is expected for a new anticancer lead compound. The utilization of microbial fermentation products as anticancer agents is advantage for its ability to produce bioactive products in control conditions at the laboratory and industry level. However, further investigations on the structure and mechanism of action of the bioactive compounds needs to be elucidated.

5.5 Chemical characterization

The chemical characteristics of the extracted metabolites were analyzed using IR and GC-MS spectrum which revealed that fraction 3 comprised of compounds such as 1-Hexadecene, E-15-Heptadecenal, 1-Octadecene, 4, 7, 10, 13, 16, 19-Docosohexaenoic acid (DHA) and 1-Docosene and fraction 5 has 9-Eicosene (E) -, 1-Octadecene and 1-Docosene. These compounds are already reported as antibacterial, antifungal and anticancer agents. Lim et al., (2010) demonstrated that growth inhibition and apoptosis
was achieved both \textit{in vitro} and \textit{in vivo} in head and neck cancer cells after exposure to H31, a metabolite from the marine \textit{Bacillus} species.

According to Mou \textit{et al.}, (2013), Hexadecene obviously affected the yields of palmarumycins C2 and C3 in \textit{Berkleasmium sp. Dzf12} liquid culture. Upon addition of 1-hexadecene at 10\% on day 6 of culture, the maximal yields of palmarumycins C2 and C3 were obtained as 0.40 g/L and 1.19 g/L, which were 40.00 fold and 59.50 fold in comparison with those of the control 0.01g/L and 0.02 g/L, respectively. Thus, 1-hexadecene addition should be viewed as an effective strategy for palmarumycin C2 and C3 production in liquid culture of the endophytic fungus \textit{Berkleasmium sp. Dzf12}. The previous results demonstrated that both palmarumycins C2 and C3 showed good antibacterial, antifungal and algicidal activities (Krohn \textit{et al.}, 1994). Previous investigations also reported that the compounds such as 1-Octadecene, 1-Heptadecane, 1-Octadecene present in both algae and higher plants are responsible for their anticancer, antioxidant and antimicrobial activities (Lee \textit{et al.}, 2007; Mishra and Sree, 2007).

Another important metabolite obtained in the present study is DHA. Docosahexaenoic acid (DHA) is a polyunsaturated fatty acid composed of 22 carbon atoms and six double bonds. Because the first double bond, as counted from the methyl terminus, is at position three, DHA belongs to the so-called $\omega$-3 group. In recent years, DHA has attracted much attention because of its beneficial effect on human health. At present, fish oil is the major
source of DHA, but alternatively it may be produced by use of microorganisms. Marine microorganisms may contain large quantities of DHA and are considered a potential source of this important fatty acid. The present study also proves that bacterioneuston can also produce DHA.

Microbial oil or single-cell oil (SCO) production is a relatively new concept, first proposed in the twentieth century (Ratledge 2001). Microorganisms, in particular the marine algae and fungi, are thought to be the primary producers of ω-3 PUFAs in the marine food chain. Although marine fish and mammals appear to have some capacity for de novo biosynthesis of ω-3 PUFAs, the majority of the PUFAs in their body originate from their diet (Ackman et al., 1964). Currently, the production of DHA by marine microorganisms is the subject of intensive research and increasing commercial attention (Barclay et al., 1994; Kyle 1996, 1997; Ratledge 2001; de Swaaf 2003). The present study showed that marine bacterioneuston can also be used as a good source of DHA.

In a previously published in vitro studies, 5 breast cell lines were used covering distinct receptor expression phenotypes: MDA-MB-231 (ER- PR- Her2-), SK-BR-3 (ERPR-Her2+), MCF7 (ER+ PR+ Her2-), MDA-MB-361 (ER+ PR- Her2+), and MCF10AT (ER+, PR isoform B but not A, Her2 variable). It is found that SK-BR-3, an ER-/Her-2+ cell line, responded synergistically to the DHA + CCM (Curcumin) combined treatment (Altenburg et al., 2011).
The overexpression of survivin has been reported in nearly all human cancers, including breast cancer (Fukuda and Pelus, 2006; Kennedy et al., 2003; Zhao et al., 2006). Siddique et al (2013) reported combined treatment of DHA + CCM caused almost a 50% reduction in survivin expression in MCF-7 cells. Disrupting survivin expression or function in cancer cells has been shown to decrease cell proliferation by enhancing apoptosis. Survivin has been considered an effective target for anticancer strategies in several preclinical and early-phase clinical trials (Doolittle et al., 2010). Thus, DHA is not only an important constituent of diet but also a better anticancer agent especially against breast cancer cells.

The data from these in vitro studies is consistent with the previously reported studies. In addition, we also report the production of DHA by bacterioneuston B. subtilis. The in vivo studies and clinical trials have to be investigated further for the reliability of the metabolites as a potential candidate for commercial drugs.