5.4 DISCUSSION

5.4.4 Cytotoxicity of bacteriocin from *Lactococcus garvieae* by MTT Assay.

Lactic acid bacteria are used as probiotics, antibiotics and anticancer agents to also extensively use against many diseases. Present study was therefore, aimed at investigating *in vitro* efficacy bacteriocin from *L. garveiae* in inhibiting growth of cancer cells. *Lactococci* have been recommended as GRAS biotherapeutic agents for cure as well as prevention of human gastrointestinal and bovine mastitis pathogens.

*In vitro* cytotoxicity assays are useful to define basal cytotoxicity, indicating the intrinsic ability of a compound to cause cell death or loss of viability as a consequence of damage to several cellular functions (Fotakis and Timbrell, 2006). The MTT assay is a commonly used assay to detect early toxicity (Fotakis and Timbrell, 2006), and it is based on a reduction of a water-soluble tetrazolium salt (MTT) to an insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenases in metabolically active cells (Mosmann, 1983). According to Berridge and Tan (1993), the reduction of MTT could also be mediated by NADH or NADPH within the cells but outside of mitochondria. The MTT assays indicated that both bacteriocins decreased viability of Vero cells in a concentration-dependent manner.

In the present study bacteriocin from *Lactococcus garvieae* showed cytotoxicity against MDA-MB - Human adenocarcinoma, mammary gland at a bacteriocin concentration of 10, 20 and 30 μg/ml showed optical density of 0.187, 0.386 and 0.598 respectively, and no cell lysis was observed at 10 μg/ml; although, there was 25% and 50 % cell lysis was noticed at a concentration of 20 and 30 μg/ml and no cell lysis in control cell line was observed. The IC 50 value of bacteriocin against MDA-MB - Human adenocarcinoma was found to be 30 μg/ml.
Further, the cytotoxicity of bacteriocin was similarly carried out against LNCap-FGC - human carcinoma Prostate at a bacteriocin concentration of 10, 20 and 30 μg/ml which showed optical density of 0.291, 0.571 and 0.722 respectively, there was 25, 50 and 75 % cell lysis was noticed at a concentration of 10 and 20 and 30 μg/ml of bacteriocin respectively. No cell lysis in control cell line was observed showing O.D value of 0.175. The IC 50 value of bacteriocin against LNCap-FGC - Human carcinoma Prostate was found to be 20 μg/ml.

Similarly, the convincing evidence suggesting that the antimicrobial peptides or bacteriocins produced by lactic acid bacteria inhibit growth of cancer cells (Cornut et al., 2008). Inhibition of cell proliferation by colicins (Chumchalova et al., 2003), microcin (Hetz et al., 2003), pediocin (Beaulieu et al., 2004) and pyocin (Abdi-Ali et al., 2004) has been established in breast carcinoma, breast adenocarcinoma, osteosarcoma, leiomyosarcoma, fibrosarcoma, T cell lymphoma, cervix carcinoma, Burkitt lymphoma, pulmonary carcinoma, colon adenocarcinoma, lymphoblastic leukemia, and hepatocarcinoma. Antineoplastic activity of pyocin has been established against mouse hepatocarcinoma and lymphoblastic leukemia using HepG2 and Im9 cell lines, whereas human fetal foreskin fibroblast was unaffected (Farkas-Himsley et al., 1976). Its uptake is possibly mediated by iron related receptors in bacterial cells (Baysse et al., 1999) and transferrin receptors in mammalian cells (Farkas-Himsley et al., 1986). This mechanism is reinforced by the fact that iron deprivation stops cell division in G1/S and leads to apoptosis in some neoplastic cell lines (LeNT et al., 2002). However, detailed in vivo investigation is required on potential use of pediocins as therapeutic agents or prophylactic compounds.

The cytotoxic effect on cancerous cells from human origin was also reported earlier (Farkas-Himsley et al., 1975). The uniqueness of the bacteriocins lies in their
interaction with the cell surface without penetrating the target cells, yet affecting cell division and DNA synthesis (Jayawardene et al., 1969) Bacteriocins are highly specific in their membrane interaction which is related to the unique receptors found in different bacterial species or types (Nomura et al., 1967).

The present studies have shown cytotoxicity of bacteriocin against cancerous cell lines MDA-MB - Human adenocarcinoma, mammary gland, LNCap-FGC - Human carcinoma Prostate cell line at concentration of 10, 20 and 30 μg/ml which was demonstrated through the induction of cytotoxicity in these cancer cell lines. In future, this information could be integrated and exploited to fully explore the suitability of Lactococcus garvieaes bacteriocin as in vivo therapeutics.

5. 4. 5 Determination of apoptotic properties of bacteriocin in PC3 cell line by 4', 6- diamidino-2-phenylindole (DAPI) staining.

The role of apoptosis in normal physiology is as significant as that of its counterpart, mitosis. It demonstrates a complementary but opposite role to mitosis and cell proliferation in the regulation of various cell populations. It is estimated that to maintain homeostasis in the adult human body, around 10 billion cells are made each day just to balance those dying by apoptosis (Renehan et al., 2001), and that number can increase significantly when there is increased apoptosis during normal development and aging or during disease.

Apoptosis is critically important during various developmental processes. As examples, both the nervous system and the immune system arise through overproduction of cells. This initial overproduction is then followed by the death of those cells that fail to establish functional synaptic connections or productive antigen specificities, respectively (Nijhawan et al., 2000; Opferman and Korsmeyer, 2003). Apoptosis is also necessary to rid the body of pathogen-invaded cells and is a vital
component of wound healing in that it is involved in the removal of inflammatory cells and the evolution of granulation tissue into scar tissue (Greenhalgh, 1998). Dysregulation of apoptosis during wound healing can lead to pathologic forms of healing such as excessive scarring and fibrosis.

Apoptosis is also needed to eliminate activated or auto-aggressive immune cells either during growth in the essential lymphoid organs (bone marrow and thymus) or in peripheral tissues (Osborne, 1996). The double-stranded DNA DAPI has an absorption maximum at a wavelength of 358 nm (ultraviolet) and its emission maximum is at 461 nm (blue). Therefore, for fluorescence microscopy DAPI is excited with ultraviolet light and is detected through a blue/cyan filter. The emission peak is fairly broad (Kapuscinski et al., 2013) DAPI will also bind to RNA, though it is not as strongly fluorescent. Its emission shifts to around 500 nm when bound to RNA. (Scott et al., 2009; Kapuscinski et al., 2013).

In the present study the cytotoxicity of bacteriocin was carried out at five concentrations viz. 50, 25, 12.5, 6.25 and at 3.125 μg/ml against PC3 human cancer cell line that showed the 17.72, 12.64, 8.69, 6.09 and 3.66 % cytotoxicity. The cytotoxicity of bacteriocin was calculated for its CTC 50 value and which depicted that >50 μg/ml was required to inhibit 50 % of cells. The bacteriocin that showed good cytotoxic effect at higher concentration in comparison to standard doxorubicin at 2 μg/ml was choosen for apoptosis activity.

The bacteriocin at 50 and 25 μg/ml was used for induction of apoptosis in PC3 human cancer cell line and standard apoptotic drug doxorubicin was used at concentration of 2 μg/ml. The bacteriocin showed apoptosis in cancer cells which was depicted though DAPI, a nuclear staining method. The cancer cells upon bacteriocin treatment showed cell plasma membrane blebbing, cell shrinkage, no
discharge of cellular components, the cells are smaller in size, the cytoplasm is dense and may be the organelles are more tightly packed.

The similar work was reported by Vogt et al., (1842) described the principle of apoptosis which shows it as a programmed death of cells, which may occur even in multicellular organisms. Various biochemical changes such as cell membrane damage, cell shrinkage, nuclear fragmentation, chromatin condensation and genetic DNA fragmentation take place during apoptosis. DNA fragmentation takes place at the end of apoptosis, which includes activation of calcium and magnesium dependent nucleases that degrade genomic DNA of susceptible cells. Currently used anticancer drugs have been shown to induce apoptosis in susceptible cells (Denicourt et al., 2004). Nuclear DNA of cells that have entered in the phase of apoptosis cell lines exposed to 25 μg/ml of rec-pediocin and native pediocin CP2 was 2.147% and 10.736% respectively, whereas that of HepG2 cell lines was 5.521% and 1.226% respectively. HeLa cells exhibited less degree of sensitivity towards rec-pediocin when compared to other cancerous cell lines of spleen lymphoblast, hepatocarcinoma and mammary gland adenocarcinoma.

A series of studies have provided convincing evidence suggesting that the antimicrobial peptides or bacteriocins produced by lactic acid bacteria inhibit growth of cancer cells (Cornutet et al., 2008). Inhibition of cell proliferation by colicins (Chumchalova et al., 2003), microcin (Hetz et al., 2002) pediocin (Beaulieu et al., 2002) and pyocin (Abdi-Ali et al., 2004) has been established in breast carcinoma, breast adenocarcinoma, osteosarcoma, leiomyosarcoma, fibrosarcoma, T cell lymphoma, cervix carcinoma, Burkitt lymphoma, pulmonary carcinoma, colon adenocarcinoma, lymphoblastic leukemia, and hepatocarcinoma.
Therefore, the present study showed that the bacteriocin can be used as a potent anticancer agent and can also be used in preparation of anticancer drug formulations.