ANTICANCER ACTIVITY OF H. pinifolia
Cancer is a dreadful human disease, increasing with changing life style, nutrition, and global warming. Cancer treatments do not have potent medicine and currently available drugs are causing side effects in some instances. In this context, a variety of ingredients of traditional medicines and herbs are being widely investigated in several parts of the world to analyze their potential as therapeutic agents (Bethan et al., 2003; Nadejda et al., 2007; Kaur et al., 2008). Most of the anticancer drugs currently used in chemotherapy are cytotoxic to normal cells and cause immunotoxicity which affects not only tumor development, but also aggravates patient’s recovery. The discovery and identification of new antitumor drug with low side effects on immune system has become an essential goal in many studies of immunopharmacology (Xu et al., 2009) and attention has been paid to natural compounds in plants, marine organism and microorganisms. Regarding the low side effects of plants and other natural compounds, scientists are interested in working on them to find new medicines. Over 60% of the currently used anticancer agents are derived from natural sources (Cragg and Newman, 2003; Balunas and Kinghorn, 2005; Cragg and David, 2005).

Breast cancer is one of the most common malignancies which affect women worldwide especially in western countries (Coleman and Tsongalis, 2002; McPherson et al., 2000). It is both genetically and histopathologically heterogeneous, and the mechanism underlying breast cancer development remains largely unknown (Hedenfalk et al., 2002). Development of breast cancer involves several types of genes that need to be activated or inactivated in order to promote malignancy (Ingrasson, 2001). A major problem with present cancer chemotherapy is the serious deficiency of active drugs for the curative therapy of tumors (Valeriote et al., 2002; Kinghorn et al., 2003). For
thousands of years, natural products have played an important role throughout the world in treatment and prevention of human diseases (Chin et al., 2006).

Despite enormous progress in the field of organic chemistry, currently 25% of all the drugs are derived from natural sources. This is more significant with regard to anticancer drugs in which more than 80% are plant-derived compounds (CRC, 1982). Finding anticancer agents from plant sources started in the earliest 1950s with the discovery and development of vinca alkaloids, vinblastine and vincristine and the isolation of the cytotoxic podophyllotoxins (Cragg and Newman, 2004). The toxicity associated with the conventional cancer chemotherapy arise primarily from the lack of specificity for tumor cells. Majority of the currently available anticancer drugs are designed to have selective toxicity towards rapidly dividing cells (Valeriote and Putten, 1975). This leads to a low therapeutic index which results in unacceptable damage to normal organs and consequently put limitation on the dose of the drug that can be administered (Deonarain and Epenetos, 1994). For example the use of anthracycline antitumor antibiotics especially doxorubicin which have a broad-spectrum of activity, are hampered by their severe dose limiting due to the cumulative cardiotoxicity (Collier and Neidle, 1988). Several approaches are being considered to handle this problem and to improving the effectiveness and tumor cell specificity of drugs in treatment of cancer. One of these methods involves the use of monoclonal antibodies which are quite expensive and their uses are time consuming.

Certain algae have long been used in traditional Chinese herbal medicine in the treatment of cancer (Yamamoto et al., 1984). Some metabolites such as bromophenols, carotene and steroids were isolated and purified from marine algae were demonstrated
for their antiproliferative activity (Xu et al., 2004). Tu et al., (2008) evaluated the antitumor activity of teriterpenoid fractions from the rhizomes of Astilbe chinensis in tumor bearing mouse. It significantly inhibited the growth of mice transplantable tumor and remarkably increased splenocytes proliferation, natural killer cells activity and the level of interleukin-2 secreted by splenocytes in tumor-bearing mice. The discovery and identification of new antitumor drug with low side effects on immune system has become an essential goal in many studies of immunopharmacology (Xu et al., 2009).

Numerous macro algae have shown potent cytotoxic activities and certain authors (Mayer and Gustafson, 2006; Smit, 2004) have suggested the consumption of algae as a chemo-preventive agent against several cancers. Ulvan, a sulfated polysaccharide, extracted from Ulva lactuca has shown cytotoxicity against human colon cell line (Kaeffer et al., 1999). Carrageenan has been found to stimulate lecithin dependent cell mediated cytotoxicity against HEp-2 human epipharynx carcinoma cells (Perl et al., 1983). Fucoidans isolated from Sargassum thunbergii and S. kjellmanianum have proven for antitumor activity (Zhuang et al., 1995; Itoh et al., 1995). Compounds of dihydroxysargaquinone and sargatriol from Sargassum tortile and diterpene from S. crispum are known for their cytotoxic activities (Numata et al., 1991; Ayyad et al., 2001). Caulerpenyne of Caulerpa taxifolia has exhibited antitumour activity against HU neuroblastoma cell line by inhibiting microtubule assembly and tubulin aggregation (Barbier et al., 2001). Cytotoxicity of Sargassum polycystum against some human cancer cell lines (Ly et al., 2005) in vitro, antitumor and antiproliferative activity of Hydroclathrus clathrus (Hui et al., 2008), various brown algae viz., Scytosiphon lomentaria, Lessonia nigrescens, Laminaria japonica, Sargassum ringgoldianum, the red algae, Porphyra
yezoensis and *Eucheuma gelatinae* and the green alga, *Enteromorpha prolifera* have antitumor activity against Meth-A fibrosarcoma (Noda *et al*., 1990). Dehydrothrsiferol and halomon extracted from *Laurencia* and *Portieria hornemannii*, respectively have been tested in the preclinical phase.

According to existing literature, more than ten new experimental anti-tumor agents derived from marine sources have entered in clinical trials, including bryostatin-1, aplidine, ecteinascidin-743 (ET-743), Kahalalide F, as well as derivatives of dolastatin such as TZT-1027 and LU 103793 (Song *et al*., 2008). Tierney *et al*. (2010) stated that owing to a diverse chemical ecology, the marine organisms especially marine flora have a great promise for providing potent, cheaper, and safer anticancer drugs, which deserve an extensive investigation. The anticancer potency mechanism through which algae exert their effects is complex because of their noteworthy structural diversity, which entails multiple interactions (Peng *et al*., 2011; Maeda *et al*., 2008). An algal antioxidant-mediated mechanism (Tierney *et al*., 2010; Ye *et al*., 2008) enhances the host’s defense by increasing natural killer cell activity (Myers *et al*., 2011), activating the nonspecific immune system (Ramberg *et al*., 2010), inhibiting the cell growth in the G1 phase, inducing terminal differentiation (Mohamed *et al*., 2012), inhibiting the complex process of angiogenesis (Ganesan *et al*., 2010), down regulating the endogenous oestrogen biosynthesis and inducting of apoptosis which were hypothesized as a contributing factors in the inhibition of carcinogenesis by algae (Namvar *et al*., 2012).

Seagrasses are rich sources of secondary metabolites and are having various phytochemicals. The present study was conducted to understand the cytotoxic effect of
the crude acetone extracts of seagrass, *H. pinifolia* against normal VERO cell line and MCF7 cell line.

**Materials and Methods**

VERO and MCF-7 cell lines were obtained from National centre for Cell Sciences (NCCS), Pune. The cells were maintained in Minimal Essential Media supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO$_2$ at 37°C. The reagent such as MEM was purchased from Hi Media Laboratories, Fetal bovine serum (FBS) was purchased from Cistron laboratories Trypsin, methylthiazolyl diphenyl- tetrazolium bromide (MTT), and Dimethyl sulfoxide (DMSO) were purchased from Sisco research laboratory chemicals Mumbai. All the other chemicals and reagents were obtained from Sigma Aldrich Mumbai.

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

The anticancer activity of the samples on VERO (African green monkey kidney Normal cell line) and MCF-7 (Breast cancer cell line) was determined by the MTT assay following the method of Mosmann, (1983). Cells (1×10$^5$/well) were plated in 1 ml of medium/well in 24-well plates and were incubated at 5% CO$_2$ incubator for 72 hours. Then, various concentrations of the acetone extracts of *H. pinifolia* were added in 0.1% DMSO (Dimethyl sulfoxide) for 48 hours maintained in a 5% CO$_2$ incubator. After the removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 200µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl--tetrazolium bromide (MTT) in phosphate buffered saline solution was added. After 4 hours of incubation, 1ml of DMSO was added. Viable cells were determined by the absorbance
at 540nm in a microplate reader. Measurements were performed and the concentration required for 50% inhibition of viability (IC$_{50}$) was determined graphically. The effect of the samples on the proliferation of VERO and MCF-7 cells was expressed as the % cell viability, using the following formula:

\[
\text{% cell viability} = \frac{A_{540} \text{ of treated cells}}{A_{540} \text{ of control cells}} \times 100
\]

**Flow cytometry assay**

The breast cancer cell line MCF-7 were trypsinized and seeded in 6-well plates at a density of 3×10$^5$ cells/well and grown for 24 hours. The IC$_{50}$ concentration were added to the cells and incubated for 24 hours, DMSO was added into the control wells. The cells were trypsinized and collected in a 15 ml falcon tube and washed twice with sterile phosphate buffered saline (PBS). After washing, the cells pellet was fixed by gently adding drop by drop ice-cold 70% ethanol with simultaneous vortexing and samples were fixed for overnight at 4°C. On the day of flow cytometer analysis, samples were centrifuged for 10 minutes at 1,000 rpm. The supernatant was discarded, and the pellets were resuspended in PBS. This step was repeated twice and ethanol was completely removed from the fixed cells. Following this, the cells were then resuspended in PBS containing 0.5% Triton X-100 (Sigma-Aldrich 93443), 0.1 mg/ml RNase (Sigma-Aldrich R4642) and 40 µg/ml Propidium iodide (Sigma-Aldrich P4170) in a dark room. Triton-X and RNAase were added to permeablize the cell membrane and eliminate RNA. After 15-30 minutes of incubation at 37°C, the cells were analyzed on a flow cytometer (FACS Calibur, Becton Dickinson), equipped with an air cooled argon laser providing 15 mW at 488 nm (Blue laser) with standard filter setup. 10,000 events were collected and the percentages of each cell cycle phases were analyzed using
Cellquest Pro software (Becton Dickinson, USA). The percentage of population containing apoptosis cells was calculated.

**Propidium iodide staining**

Examination of the nuclear chromatin morphology was performed following the method of Chow et al., (1995) and Balasubashini et al., (2006). MCF-7 cells were grown on a 6-well plates with cover slip, and treated IC_{50} concentrations of *H. pinifolia* acetone extracts for 48 hrs. After incubation, the treated cells were fixed with 70% ethanol and left at 4°C overnight. Then cells were rinsed with phosphate buffered saline (PBS) and stained with Propidium iodide (1mg/ml) solution containing 0.05% Triton X and RNAase (1mg/ml) and incubated for 30 minutes. After incubation, the Propidium iodide stained DNA was observed using an LSM Meta 510 Confocal Scanning Microscope (Carl Zeiss, Germany).

**Result**

**In vitro cytotoxic analysis by MTT assay**

To study the anticancer effects, the acetone extracts of *H. pinifolia* was tested for its effect on inhibition of cell growth against two cell lines i.e., normal VERO and MCF-7 over a concentration range of 7.8-1000 µg/ml to determine their potency (IC_{50} ie,50% inhibition of cell growth). Assay was performed *in vitro* on exponentially growing cells. The activity was evaluated by measuring the levels of surviving cell after incubation with the test samples, using the MTT assay and is represented in Figs. 1 & 2. It was noticed that in the normal cell line i.e., VERO cells the IC_{50} was 500 µg/ml and that of the MCF-7 cell line it was 62.5 µg/ml. It was seen that the negative control had
100% viability of cells in both the cell lines. The degree of the MCF-7 cell inhibition showed a dosage-dependent relationship with the treatment with the acetone extracts of *H. pinifolia*.

The increasing concentration of the acetone extracts of *H. pinifolia* on the MCF-7 cell line reduced the cell viability. The increasing concentration of the extract on the VERO did not show significant reduction in the cell viability when compared to the MCF-7 cell line. At higher concentration (1000µg/ml), the VERO cell line showed cell viability of only 41.7% whereas at the same concentration the MCF-7 cell line showed only 10.1% cell viability. This showed that the crude extract of *H. pinifolia* showed cytotoxic effects to the MCF-7 cells rather than the VERO cells (Plates 1 and 2).

**Cell cycle analysis by flow cytometry**

Cell cycle is a common feature of cells that are undergoing terminal differentiation and defective proliferation. To determine the effect of the crude acetone extract of *H. pinifolia* on the cell cycle, the MCF-7 cells were treated with the IC$_{50}$ levels of the extract (determined by the MTT assay) for 24 hours. The cells were stained with PI and the DNA contents were analyzed by flow cytometry. The proportion of cells in different phases of the cell cycle was analysed by the histogram statistics. The relative DNA content in untreated and treated MCF-7 cells was represented as three distinct stages within the interphase of the cell cycle, called G0/G1, S and G2/M phases. From the results, there were no changes in the DNA content in each phase of the untreated cells. Untreated MCF-7 cells showed a normal cell cycle distribution of approximately 79.20% in G0/G1 phase, 12.83% in S phase and 0.09% G2-M phase. For the MCF-7 cell line which was treated with the acetone extract of *H. pinifolia*, DNA histograms
exhibited a prominent decrease of G0/G1 cell population. The size and granularity of cell was measured by Forward angle scatter and Side scatter. There was an increase in sub G0/G1 from a control value of 6.39 to 6.50 which indicated that apoptosis had started. In the present study, there was an increase in the side scatter (SSC) which revealed that there was an increase in the granularity when compared to the control cells. Decrease in the forward scatter was observed which showed the presence of lower cell size.

In the S phase, the control value of 12.83% increased to 13.21% which showed that there was arrest in this phase. This increase was seen due to the decreasing G2M phase after 24 hours. This shows that apoptosis occurred in the MCF-7 cell line (Fig. 3 and 4).

**Propidium iodide staining**

The IC$_{50}$ concentration of the acetone extracts of *H. pinifolia* treatment contained more apoptotic cells when compared to control monolayer of cells. Characteristic features of reduced cell size and intense fluorescence of condensed nuclear chromatin at this concentration showed apoptosis (Plate 3)

**Discussion**

According to the American Cancer Society, the global burden is expected to grow as 27 million new cancer cases and 17.5 million cancer deaths due to the growth and aging of the population by 2050. Natural derivatives play an important role to prevent the cancer incidences as synthetic drug formulations cause various harmful side effects to human beings. Of the anticancer compounds extracted so far, the marine algal
contribution is 65.63%. Owing to a diverse chemical ecology, the marine organisms especially marine flora have a great promise for providing potent, cheaper, and safer anticancer drugs, which deserve an extensive investigation (Ganga et al., 2011).

Cancer chemotherapeutic agents can often provide temporary relief from symptoms, prolongation of life and occasionally complete remission. A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damages to normal cells. This ideal situation is achievable by inducing apoptosis in cancer cells. Chemopreventive agents comprise diverse groups of compounds with different mechanisms of action with ultimate ability to induce apoptosis. Understanding the mode of action of these compounds should provide useful information for their possible applications in cancer prevention and perhaps in cancer therapy (Taraphdar et al., 2001; David, 2004).

Cell cycle modulation by various natural and synthetic agents is gaining widespread attention in recent years. Given that disruption of cell cycle plays a crucial role in cancer progression, its modulation by phytochemicals seems to be a logical approach in control of carcinogenesis (Singh et al., 2002). The ability of a substance to affect specific phases of the cell cycle may provide clues as to its mechanism of action. A reduction in cell growth and an induction in cell death are two major means to inhibit tumor growth (Firestone and Bjeldanes, 2003). Apoptosis is one of the important pathways through which chemo preventive and chemotherapeutic agents inhibit the growth of cancer cells (Ming et al., 2012).
In the present study, the acetone extract of *H. pinifolia* was used to check whether it had the capability of inducing cytotoxic effects on the VERO and MCF-7 cell lines. In the MTT assay method both the cell lines were used by using the concentration range of 7.8-1000 µg/ml and the IC<sub>50</sub> concentration were determined. The IC<sub>50</sub> concentration of MCF-7 was 62.5µg/ml. It was noticed that the crude extract of *H. pinifolia* had toxic effects on the MCF-7 cell line but the normal VERO cell line was not affected. The IC<sub>50</sub> values of the present investigation was lesser than the findings of Girija *et al.*, (2013b) who reported the IC<sub>50</sub> value of ethyl acetate fraction of *H. pinifolia* collected from the vellar estuary on MCF-7 cell line of 66.68µg/ml. The invitro cytotoxicity was meant to determine the IC<sub>50</sub> of the crude sample towards to the cells.

It is evident that the acetone extracts of *H. pinifolia* exhibited less prominent antiproliferative activity on the Vero cell line. The extracts mediated antiproliferative activity is limited to the cancer cell lines rather than the normal cell lines. This indicates that the specific inhibitory effect may be due to the apoptosis-inducing ability of the acetone extracts of *H. pinifolia* in response to the defective gene expression in cancer cell lines rather than the normal cell line. With the significant antiproliferative activity of the extracts of seagrass against MCF-7 cancer cell lines, the mechanisms of action could, possibly, be due to the dose-dependent apoptosis-inducing ability, by necrosis of cancer cell lines, by enhanced neoplastic transformation followed by apoptosis or by any other mechanisms related to epigenetic and signal transduction pathways. Phytochemicals such as vitamins (A, C, E, and K), carotenoids, terpenoids, flavonoids, polyphenols, alkaloids, tannins, saponins, pigments, enzymes and minerals have been found to elicit anticancer activities. These metabolites obstruct various hormone actions

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and metabolic pathways associated with the development of cancer (Gacche et al., 2011, Lu et al., 2008).

The cytotoxicity of natural products is based on the presence of antitumor metabolites. Bioactive cytotoxic compounds have been found in marine algae. Several sulfated polysaccharides separated from algae have shown antitumor, anticancer, and antimetastatic activities in mice (Coombe et al., 1987). Antitumor activity has also been noted with the macro algae *Sargassum stenophyllum* (Dias et al., 2005). In addition, the hydroquinone diterpene from *Cystoseira mediterranea* has been shown to have an inhibitory effect on mitotic cell division (Francisco et al., 1985).

The IC$_{50}$ of the present study was even lower than the methanolic extract and partition fractions of the seaweed *S. swartzii* of Asaluye-Niband protected marine area in Persian Gulf (Mahnaz et al., 2010). The hexane fraction showed IC$_{50}$ value of 99.9±19.38 µg/ml against Caco-2 cell line and methanolic extract of *S. swartzii* exhibited cytotoxic activity which showed IC$_{50}$ of 205.21±84.1 µg/ml against T47D cell line. The observation of cytotoxic activity level in the present study was higher when compared with butanol extract of *A. protuberus* of the marine sediments of south Indian coastal belt that showed cytotoxicity against Hep2 cells with IC$_{50}$ less than 125 µg/ml (Mathan et al., 2011). Extract of fish *Pollachius virens* and *Scophtalamus rhombus* collected from North Atlantic showed specific inhibition of mitochondrial activity with CC$_{50}$ values (50% cytotoxic concentration) around 500 mg/ml on the 3T3-cell line (Hellio et al., 2002) which has very lower activity when compared to the present study.
The MTT colorimetric assay (Martine et al., 2008; Suganumak and Saikawa, 2003) is based on the ability of metabolically active cells to convert the pale yellow MTT to a blue formazan product, which is quantifiable spectrophotometrically. The MTT assay is a method to measure the effectiveness of the sample in inhibiting the biological or biochemical function (Cheng and Prusoff, 1973). This quantitative measure indicates how much of a particular sample is needed to inhibit cancer cells growth by half and this is very useful in pharmacological research. Moreover, this biological assay, allows judgment to be made whether the compound is active or not. The usage of MCF-7 breast cancer cell lines is widely used nowadays in numerous researches for the anticancer properties. MCF-7 cells are the most commonly used model of estrogen positive breast cancer. This cell line has been originally established in 1973 at the Michigan cancer foundation from a pleural effusion taken from a woman with metastatic breast cancer (Soule et al., 1973) and since then MCF-7 cells have been widely distributed in laboratories throughout the world resulting in the production of different cellular stocks. The VERO cell is a normal mammalian cells extracted from African green monkey kidney which is generally used in pharmaceutical research (Yasumura and Kawakita, 1963). The acetone extract of *H. pinifolia* had no significant cytotoxic effect on normal VERO cells, suggesting that this selective killing effect of the extract against actively proliferating cells could be exploited in developing this compound as a potential antitumorigenic agent.

The mechanism of apoptosis was analysed with the IC\textsubscript{50} concentrations of the acetone extracts of *H. pinifolia* by flow cytometry and the histogram statistics revealed that there was increase in the sub G0/G1 and in the S phase, the control value of
12.83% increased to 13.21% which showed that there was arrest in this phase. Patil et al., (2012) reported the effect of *E. agallocha* extract on the cell cycle progression in human lung carcinoma cell lines A549 (p53<sup>+/+</sup>) and H1299 (p53<sup>-/-</sup>) with concentration of 2 × IC<sub>50</sub> for 24 hours. After treatment with the extract for 24 hours, A549 cells showed increased DNA contents of the sub-G1 phase as compared with the control. For H1299 cells, the cells with sub-G1 DNA content increased from 2% in control to 3% in 24 hours treated with *E. agallocha* 2×IC<sub>50</sub> treated sample. The G1 or G0 fraction changed from 54% to 58% in H1299 cells indicating G1 arrest. Several mangroves species such as *Acanthus illicifolius*, *Bruguiera sexangula*, *Morinda citrifolia*, *Terminalia catappa* and *Ecteinascidia turbinata* have been shown to produce compounds that show strong activity against a variety of carcinomas, melanomas and lymphomas (Jongsuvat, 1981; Goh and Jantan, 1991; Linuma et al., 1994; Hirazumi and Furasawa, 1999; Bandaranayake, 2002).

Since the concept of apoptosis was reported by the observation of (Kerr et al., 1972), many techniques such as DNA ladders, flow cytometry, in situ nick translation analysis and so forth have been developed as markers of apoptosis. However, morphological changes still provide the most reliable criteria for recognizing apoptotic process (Harmon and Allan, 1997; Liu et al., 1999). Induction of apoptosis is a useful approach in cancer therapies. In apoptotic cells, several cellular and molecular biological features, such as cell shrinkage, DNA fragmentations, and activation of the caspase cascade, are exhibited (Germain et al., 1999). Apoptotic cells can be recognized by their diminished staining ability with DNA specific fluorochromes such as Propidium iodide, DAPI, Acridine orange (AO), or Hoechst dyes, due to DNA
degradation and its subsequent leakage from the cell. Of these existing dyes propidium iodide proved to be an excellent probe to distinguish live, necrotic, early and late-apoptotic cells rather than other staining (Darzynkiewicz et al., 1992). It is to be noted that Propidium Iodide stains are being used more often than other nuclear stains because it is economical, stable and a good indicator of cell viability (Fried et al., 1976; Bacso et al., 2000).

In the present study, the MCF-7 cell lines incubated with the test extract was stained with propidium iodide stain. It was observed that there was reduced cell size and nuclear condensation of cells by the acetone extracts of *H. pinifolia* than the control cells. Farideh et al., (2013) reported that the cytotoxicity of seaweed *Sargassum muticum* was evaluated by growth inhibition. When the growth inhibited cells were stained with AO/PI and Hoechst 33342, apoptotic cell death was observed in time and dose dependent manner. Untreated cells show a diffuse green fluorescence, while in apoptotic cells condensed chromatin material resulted in clumps of intense green fluorescent spots within the cells. The characteristic condensation pattern observed were the crescent shape at the nuclear periphery and the more numerous round clumps.

The present study suggests that the acetone extract of *H. pinifolia* had cytotoxic property and further the compounds can be isolated for the possible source of antitumour agents. The compounds present in this extract may be a promise for use as a cancer chemotherapeutic agent in the future and can be used as scientific evidence to support anticancer properties of *H. pinifolia.*
Fig. 1: MTT assay of acetone extract of *H. pinifolia* on VERO cell line

Fig. 2: MTT assay of acetone extract of *H. pinifolia* on MCF-7 cell line
Fig. 3: Flow Cytometry of control on MCF7 cell line
Fig. 4: Flow cytometry of IC₅₀ concentration of acetone extract of *H. pinifolia* on MCF 7 cell line
Plate 1: MTT assay of different concentrations of acetone extract of *H. pinifolia* in VERO cell line
Plate 2: MTT assays of different concentrations of acetone extracts of *H. pinifolia* in MCF-7 cell line.
Plate 3: Propidium iodide staining