8. ANTI-CANCER ACTIVITY OF 5-OXA-6-AZASPIRO [3.4] OCT-6-ENE DERIVED FROM THE HALOTOLERANT FUNGUS ALTERNARIA ALTERNATA

8.1. Introduction

Both flora and fauna of marine ecosystem is increasingly exploited worldwide for the findings of new pharmaceuticals. In relation to a competitive aptitude developed in many, diverse and extreme environments, microorganisms are able to produce secondary metabolites with cytotoxic and antiproliferative properties that are valuable in the perspective of antitumor drug discovery (Nicoletti et al., 2008).

Cancer is a malignant neoplasm, comprises a broad group of diseases involving unregulated cell growth. In cancer, cells divide and grow uncontrollably, forming malignant tumors, and invade nearby parts of the body. The cancer also spread (metastasis) to more distant parts of the body through the lymphatic system or bloodstream. Not all tumors are cancerous, benign tumors does not invade neighboring tissues and do not spread throughout the body (CR, 2012). Cancers are primarily an environmental disease with 90-95% of cases attributed to environmental factors and 5-10% due to genetics (Anand et al., 2008). It also caused by both internal factors (such as inherited mutations, hormones and immune conditions) and environmental/ acquired factors (such as tobacco, diet, radiation and infectious organisms).

Cancer continues to be a worldwide killer, despite the enormous amount of research and rapid developments seen during the past decade. According to recent statistics, cancer accounts for about 23% of the total deaths after heart diseases
By 2020, the world population will be expected to increase 7.5 billion, of this number approximately 15 million new cancer cases will be diagnosed and it may leads to death of 12 million (Brayand and Møller, 2006). These compiled data shows that the number of male, female and the total cancer patients in 2004 were 390809, 428545 and 819354 respectively.

There are over 200 different known cancers that afflict humans. Breast cancer is the second most common type of cancer after lung cancer and the fifth most common cause of cancer death (WHO, 2007). It was mainly caused by mutations and it has been experimentally linked to estrogen exposure (Cavalieri et al., 2006). Worldwide, breast cancer accounts for 22.9% of all incidents (excluding non-melanoma skin cancers) in women. In 2008, breast cancer caused 458,503 deaths worldwide (13.7% of cancer deaths in women) (WCR, 2008). In India, most frequently observed cancers are lungs, breast, colon, rectum, stomach and liver (Nandakumar, 1990; Rao and Ganesh, 1998; Murthy and Mathew, 2004). According to the reports, breast cancers have badly attacked women population in India. A survey carried out by Indian Council of Medical Research (ICMR) in the metropolitan cities viz. Delhi, Mumbai, Bangalore and Chennai from 1982 to 2005, has shown that the frequency of breast cancer have doubled. Over the years, the prevalence of breast cancer in India has steadily increased and as many as 1,00,000 new patients are being detected every year (Michael and Jernal, 2003; Yip et al., 2006). Breast cancer is set to overtake cervical cancer as the most common cancer in women in India in 2020 (Shetty, 2012).
Oral cancer found to be fourth common type of malignancy after lung, stomach and liver in males and listed fifth in cancer disease after cervix, breast, stomach and lung cancer in females (Park, 1997). Oral or mouth cancer, that found in the oral cavity (the mouth area) and oropharynx area (the throat area at the back of the mouth). It is a subtype of head and neck cancer, and refers to any cancerous tissue growth located in the oral cavity (Lozano, 2012). Globally, as of 2010, 1, 24,000 people died of oral cancer up from 82,000 in 1990. The death rate for oral cancer is higher than cervical cancer.

Oral cancer is very common in India (ICMR, 1992) and it may arise as a primary lesion originating in any of the oral tissues, by metastasis from a distant site of origin, or by extension from a neighboring anatomic structure, such as the nasal cavity. There has been a substantial increase in the incidences of oral sub-mucous fibrosis, which further increased the incidence of the oral cancer (Gupta et al., 1998). It is mainly caused by the use of tobacco; it affects oral cavity, pharynx, oesophagus, larynx, lungs and urinary bladder (Bobba et al., 2003).

A large number of chemo-protective agents (1,500 anticancer drugs are in active development with over 500 of the drugs under clinical trials) are used to kill/suppress the cancer cells, which always leads to produce toxic side effects that limits their extensive usage. Much progresses needs to be made to overcome the problems of resistance and toxic side effects of existing cancer chemotherapeutic agents (Chabner et al., 2005; Devita et al., 2008).
There are two main disadvantages of chemoprotective agents. They are,

- Anticancer agents (chemicals) cannot differentiate between cancer and normal cells and it leads to death of actively dividing normal cells.
- Another limitation is that the cancer cells may become resistant to chemotherapy drugs after prolonged administration.

Natural compounds have substantial structural diversity and frequently afford new mechanisms of biologically active anticancer agents that has been discovered and developed primarily by many pharmaceutical industries (Cragg et al., 2005).

Many pharmaceutical approaches has developed for culturing fungi that supports the production of unusual metabolites to discover new anticancer with diverse chemical structures and novel mechanism of action for new and reemerging diseases without causing any side effects (Rojas et al., 2003). Most of the structurally diverse anticancer compounds were derived from various sources of fungal species, it includes brefeldin A (Satiat-Jeunemaitre and Hawes, 1993), Cytochalasin E (Udagawa et al., 2000), Gliotoxin (Lee et al., 2001), Illudin S (Kelner et al., 2008), Irofulven (Butler, 2008), the Leptomycins, Palmarumycin (Cragg et al., 2005; Sulkowska-Ziaja et al., 2005), Terrecyclic acid A (Turbyville et al., 2005), and Wortmannin (Ui et al., 1995). Alternaria species produce a wide variety of primary and secondary metabolites, which have an varied structure and unusual partially saturated compounds like perylene and ergosta-4,6,8 (14)-22-tetraen-3-one (ETO) from Alternaria alternata (Seitz and Paukstelis, 1977). A cyclic depsipeptide such as alternaramide was also isolated from the marine-
derived halotolerant fungus *Alternaria* sp. have a cytotoxic effect against Aurora B, FLT3, IGF1-R and VEGF-R2 (Davis and Stack, 1991; Kim *et al.*, 2009).

Few reports were available on anticancer compounds derived from halotolerant fungus *Alternaria alternata*, but this fungus that reside in extreme environments are capable to produce active cytotoxic metabolites. In this context, the natural cytotoxic products derived from halophilic fungi have gained significance in the treatment of cancer.
8.2. Materials and methods

8.2.1. Cell lines

The Laryngeal squamous cancer cell line (HEp-2) and breast cancer cell lines (MCF-7) were obtained from National Centre for Cell Science (NCCS), Pune. The HEp-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), at 37°C in humidified atmosphere with 5% CO₂.

MCF-7 was grown in Eagles Minimum Essential Medium (EMEM) containing 10% Fetal Bovine Serum (FBS) at 37°C, 5% CO₂, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly and the culture medium was changed twice a week.

8.2.2. Cell treatment

The cancer cells were plated in 96 well flat bottom tissue culture plates at a density of approximately 1.2×10⁴ cells/well and allowed to attach overnight at 37°C. The medium was then discarded and cells were incubated with different concentrations of the fungal compound derived from Alternaria alternata (12.5, 25, 50, 75 and 100 µg) in HEp-2 cell lines and 2.5, 5, 10, 20 and 40 µg concentrations in MCF-7 cell lines for 48 hours. After the incubation, medium was discarded and 100µl of fresh medium was added with 10 µl of MTT (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide) (5 mg/ml). After 4 hours, the medium was discarded and 100 µl of DMSO was added to dissolve the
formazan crystals. In case of MCF-7 cell lines, 1 µl of DAPI and AO/EB were stained to view the nuclear morphology.

8.2.3. Staining

**DAPI** – (4’, 6-diamidino-2-phenylindole)

- Dilute the DAPI stock solution to 3 µM in staining buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 0.5 mM MgCl₂ and 0.1% Nonidet P-40).
- 1 µl/well of final volume was used to stain the cell for 5 minutes.

**AO/EB** – (Acridine Orange/Ethidium Bromide)

- 100X stock solution of Acridine Orange/Ethidium Bromide stain was prepared and frozen according to the protocol provided in the manual of immunological methods (Brousseau *et al.*, 1999).
- Stain the cells with 1µl/well of AO/EB for 5 minutes.

8.2.4. MTT assay

The MTT assay (Mossman, 1983) is based on the ability of live but not dead cells to reduce the yellow tetrazolium dye to a purple formazan product. 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. Then, the absorbance was read at
570nm in a microtitre plate reader. Cell survival was calculated by the following formula,

\[
\text{Viability \%} = \left( \frac{\text{Test OD}}{\text{Control OD}} \right) \times 100
\]

\[
\text{Cytotoxicity \%} = 100 - \text{Viability\%}
\]
8.3. Results

Results revealed that the 5-Oxa-6-azaspiro [3.4] oct-6-ene was found to be more effective against both HEp2 and MCF-7 cancer cell lines. The percentage of cell death was assessed using MTT assay in HEp-2 cell lines (Fig. 60). The maximum cytotoxicity was observed at 100 µg/ml concentration in HEp-2 cancer cell lines (Photoplate 10). In case of MCF-7, the cytotoxicity was maximum at 40 µg/ml (Fig. 61). The staining of AO/EB that detects apoptotic cells, green cells indicates viable cells, altered cell morphology that pointed early apoptotic cells and orange color indicates late apoptotic cells (Photoplate 11). Blue fluorescent of DAPI, stains the nucleic acid of dsDNA but no cytoplasm. The blue fluorescence increases with increasing cell death (Photoplate 12). IC₅₀ values exhibited by 5-Oxa-6-azaspiro [3.4] oct-6-ene was found to be 30 µg/ml in both MCF-7 and HEp-2 cancer cell lines.
**Fig. 60.** Cytotoxicity effect of 5-Oxa-6-azaspiro [3.4] oct-6-ene from *Alternaria alternata* against HEp-2 cell line.

**Fig. 61.** Cytotoxicity activity of 5-Oxa-6-azaspiro [3.4] oct-6-ene from *Alternaria alternata* against MCF-7 cell line.
8.4. Discussion

A number of fungal compounds have been investigated for anticancer activities which are structurally diverse compound shown to have potential anticancer activity. For instance, Irofulven as a DNA synthesis inhibitor based on the lead compound, Illudin-S, late-phase oncology clinical trials (Butler, 2008). Recently, Lodamin, a new angiogenesis inhibitor based on TNP-470, itself related to Cytochalasin E and originally isolated from *Aspergillus fumigatus* (Kusaka et al., 1994). Later, the compounds were modified using nanotechnology, has shown promising effects in murine models of a number of cancer types (Satchi-Fainaro et al., 2004). The antitumor active isolates were identified and belonged to 12 taxa out of which *Alternaria* species, are reported as producers of anticancer compounds taxol and Brefeldin-A (Strobel et al., 1996; Vurro et al., 1998).

Hypersaline environments provide an excellent source for unique and synthetic compounds that are highly participated in medicinal part. In this present investigation, the compound derived from halotolerant fungus *Alternaria alternata* showed promising cytotoxic activity this may be due to the saline or osmotic stress in high salt concentrations might activate some silent genes to produce novel secondary metabolites as reported by Koch (1993). Xin et al. (2009) reported new bioactive compounds from halotolerant fungi *Penicillium notatum* produce cytotoxic metabolites against cdc2 mutant cell line. Cytotoxic activity against MCF-7 and HEp-2 cancer cell lines with the IC$_{50}$ value of 30 $\mu$g/ml was reported in this study. Likely, Xiao et al. (2013) isolated the compounds (Ergosterol, Rosellichalasin and Cytochalasin E) from halophilic fungi- *Aspergillus* sp and it
showed the cytotoxicity activity against human colon cancer cell line RKO with IC$_{50}$ of 3.3 ± 0.5 μM. Wang et al. (2007a) suggested some new secondary metabolites from halotolerant *Aspergillus variecolor* showed activity against the P388, HL-60, BEL-7402, and A-549 cell lines with IC$_{50}$ values from 70 to 260 μg/ml.

In this context, the potential strain *Alternaria alternata*, produce unique and structurally diverse compound showed wide range of inhibitory effect on cancer cell proliferation by interference with the cell cycle and arrest at growth phase. Aly et al. (2008) reported that endophytic fungus *Alternaria* species produce the compounds Alternariol, Alternariol its monomethyl ethers, Alternariol 5-O-methyl ether, Altenusin, and Desmethylaltenusin showed cytotoxic activity. Lehmann et al. (2006) reported the compound Alternariol showed good cytotoxic activity. Wang et al. (2009) stated that ethyl acetate extract of *Alternaria raphani* showing significant cytotoxicity against the mouse cdc2 mutant cell line which may be due to the presence of altenusin-type compounds with the alternariol derivatives indicated that the presence of a lactone ring.

In this study, HEp-2 cancer cell lines were treated with 5-Oxa-6-azaspiro[3.4]oct-6-ene and showed highest activity at 100 μg concentration with IC$_{50}$ value of 30 μg/ml. This study supported by Karthick et al. (2012) suggested the endophytic fungal metabolites has an anti-tumour activity against HEp-2 cell line reveals 100 μg concentration inhibited maximum viability. Aly et al. (2008) reported that endophytic fungus *Alternaria* species with IC$_{50}$ values ranging from 1.7 to 7.8 μg/ml against L5178Y mouse lymphoma cells which may be due to the differences in IC$_{50}$ values result from a number of factors in addition to the binding
Cytotoxic Activity

of drug to the biochemical target for the cytostatic or cytotoxic response. These factors include the incubation time and cell cycle time (IC$_{50}$ values generally decrease with increasing number of cell cycles traversed by the line during the incubation period), and rates of drug uptake, efflux, and metabolism. Differences in the content of added proteins (e.g. the concentration of fetal bovine serum) can change free-drug concentrations, and differences in medium constituents can differentially alter drug stability (Finlay et al., 1986). Joel and Bhimba (2013) reported the cytotoxic activity from mangrove fungus Neurospora crassa against HEP-2 cell lines and concerned IC$_{50}$ values were found to be 125 µg/ml. Bhimba et al. (2012) reported the mangrove derived fungus Hypocrea lixii showed the best anticancer activity against both HEP2 and MCF-7 cell line. Mathan et al. (2011) reported the fungus Aspergillus protuberus isolated from marine sediments (Saprophytes) exhibited anticancer activity against HEP-2 cells.

In this present investigation, MCF-7 cell lines were treated with 5-Oxa-6-azaspiro [3.4] oct-6-ene and showed the cytotoxicity at maximum concentration of 40 µg/ml with IC$_{50}$ value of 30 µg/ml. Joel and Bhimba, 2013a reported the ethyl acetate extracts from mangrove associated fungus Meyerozyma guilliermondii showed potent cytotoxicity against HEP-2 and human breast adenocarcinoma (MCF-7) cell lines with IC$_{50}$ values of 1.25 and 0.625 µg/ml. Deng et al. (2013) suggested that the compounds from mangrove endophytic fungus Aspergillus terreus showed the in vitro cytotoxicity against MCF-7. Elavarasi (2012) studied the anticancer activity of Taxol from Mangrove derived Endophytic fungus Fusarium moniliforme against MCF-7 cell lines. Kobayashi et al. (1989) reported that the calphostin showed cytotoxic activity against MCF-7 cells.
Cytotoxic Activity

To detect the apoptotic cells, the MCF-7 cell line was stained with DAPI and AO/EB to visualize the nuclear morphology of cancer cells. Generally, AO/EB method employs a viability stain in which AO diffuses into all cells and EB is not able to diffuse across a cell membrane unless it is compromised or the cell is apoptotic. AO makes a cell green, and EB makes a cell orange. In this study, maximum apoptotic activity increases with the increasing concentration (10-40 µg/ml) showed orange in color. Live cells will appear uniformly green in the concentration of 2.5 µg/ml, early apoptotic cells will stain green and contain bright green dots in the nuclei due to the consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells incorporate with ethidium bromide and stain orange, but, in contrast to necrotic cells, the late apoptotic cells will show condensed and often fragmented nuclei. Necrotic cells stain orange, but have a nuclear morphology resembling that of viable cells, with no condensed chromatin. In this study, the cell does not showed resemblance of necrotic cells, thus the cell morphology indicates the cytotoxicity caused by apoptotic activity. In contrast to the blue-fluorescent DAPI, the cells showed blue fluorescence increases with increasing cell death, it stain the nucleic acid preferentially dsDNA, due to the associates with AT clusters in the minor groove (Kubista et al. 1987). Binding of DAPI to dsDNA produces a ~20 fold fluorescence enhancement, apparently due to the displacement of water molecules from both DAPI and the minor groove (Barcellona et al. 1990).

In the course of this study, new bioactive metabolite (5-Oxa-6-azaspiro[3.4] oct-6-ene) from halotolerant fungi Alternaria alternata was observed to produce cytotoxic metabolites against both HEp-2 and MCF-7 cell lines also
suggests the possible utilization of halophilic fungal metabolites as anticancer drug.
Photoplate 10. MTT assay of 5-Oxa-6-azaspiro [3.4] oct-6-ene at different concentration against laryngeal squamous cancer cell line (HEp-2)
Photoplate 12. MTT assay of 5-Oxa-6-azaspiro [3.4] oct-6-ene at different concentration against breast cancer cell line (MCF7) and its effects on nuclear morphology stained with DAPI.
Cytotoxic Activity

Photoplate 11. Effects on A0/EB staining of MCF7 cells

A - Viable cells; B - Early apoptotic cells; C - Late apoptotic cells