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In vitro cytotoxicity MTT assay in Vero, HepG2 and MCF -7 cell lines study of Marine Yeast

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ABSTRACT

Marine yeasts isolated from coastal mangrove ecosystem namely *Candida albicans*, *Kuraishia capsulate* and *Sacchromyces cerevisae* were screened for the cytotoxicity against a non-transformed Vero (African green monkey kidney normal cell line) and two cancer cell lines human breast carcinoma cells (HepG2), human breast carcinoma cells (MCF -7) in different concentrations (1000 to 1.953 µg/ml). Lower doses enhanced the viability of the cultured cells, MTT assay .higher doses decreased viability of the cells by 50% or more. MTT assay was used to measure the cell proliferation and survival. Amongst three yeast strains, *Sacchromyces cerevisae* showed more than 80% cell viability in Vero cell lines and were studied for further cytotoxicity against HepG2, MCF -7 cell lines respectively.

INTRODUCTION

Natural resources, such as plants, microorganisms, vertebrates and invertebrates, are an indispensable source of bioactive compounds. A large number of drugs have been developed in medicinal practice from natural products (Amador *et al*., 2003). Since the discovery and success in the treatment of penicillin, microorganisms have been especially used as a privileged source of structurally diverse bioactive agents. The therapeutic application of microbial metabolites provided the opportunity for the discovery of antibiotics (*e.g.*, penicillin, erythromycin, streptomycin, amphotericin and polyketides), immunosuppressants in transplantation (*e.g.*, cyclosporine A, FK506 and rapamycin), cholesterol lowering agents (*e.g.*, lovastatin and mevastatin) and anticancer agent *(e.g.*, daunorubicin, doxorubicin, bleomycin and pentostatin). Resveratrol decreased the cytotoxicity of formaldehyde depending on cell line and point of time, especially in case of MCF-7 cells at 24 and 72 h, Vero cells at 24 h and HepG2 cells at 48 h after treatment. Possible modes of interactions are discussed, considering the role of resveratrol in formaldehyde metabolism and also the estrogen receptor positivity of MCF-7 cells (Marcsek *et al*., 2007).Cancer appears to be a major cause of morbidity and mortality and runs in the top three causes of death worldwide, especially in the developed countries.

Chemotherapy is one of the potential treatments for prolonging the patient's life. Almost 60% of anticancer drugs are of natural origin, such as plants (*i.e*., vincristine, irinotecan, camptothecines) and microorganisms (*i.e*., doxorubicin, dactinomicines, mitomycin and bleomycin) (Grever, 2001).

Vero cells are sensitive to infection with SV-40, SV-5, measles, arboviruses, reoviruses, rubella, simian adenoviruses, polioviruses, influenza viruses, Para-influenza viruses, respiratory syncytial viruses, vaccinia, and others. HepG2 cells, which are easy to handle, retain many of the morphological characteristics of liver parenchymal cells (Knowles *et al*., 1980), and contain several enzymes responsible for the activation of various xenobiotics (Diamond et al., 1980; Sassa *et al*., 1987). In a series of experiments carried out by our group, human tumor (HT-29, SW-620, HT-1080) and endothelial (HUV-EC-C) cells were treated with various doses of resveratrol (0.1e100 mg/ml) in vitro. Cell number, apoptotic and mitotic indexes were measured 24, 48 and 72 h following treatment.

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Low doses (0.1-1.0 mg/ml) of resveratrol enhance cell proliferation; higher doses (10e100 mg/ml) induce apoptosis and decrease mitotic activity, which is reflected in changes of cell number. The induction of apoptosis is a possible explanation for the antiproliferative effect of resveratrol (Szende *et al*., 2001).Similarly, MCF-7 breast cancer cells serve as an excellent in vitro model for studying the mechanism of tumour response as well as complex relationships between binding and biological actions of hormones. (Dillon *et al*., 2010) and the ability of MCF-7 cells to process estrogen, in the form of estradiol, via estrogen receptors in the cell cytoplasm makes the MCF-7 cell line an estrogens receptor (ER) positive control cell line.

The MTT Assay (Mosmann, 1983) is a sensitive, quantitative and reliable colorimetric assay that measures viability, proliferation and activation of cells. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product that is insoluble in water. Viable cells are able to reduce the yellow MTT under tetrazolium ring cleavage to a water-insoluble purple-blue formation which precipitates in the cellular cytosol and can be dissolved after cell lysis, whereas cells being dead following a toxic damage, cannot transform MTT. This formation production is proportionate to the viable cell number and inversely proportional to the degree of Cytotoxicity. The reaction is mediated by dehydrogenases enzymes associated with the endoplasmatic reticulum and the mitochondria (Fotakis and Timbrell, 2006).

However, many chemotherapeutic drugs are presently placed in a predicament of reduced therapeutic effect due to the problem of drug-resistance (Peters *et al*. 2002). Chemotherapeutic drugs also exert toxicity to normal cells, which in turn causes the unpleasant side effects to the patients. For these reasons, research and development of new classes of anticancer agents which exhibit efficient and selective toxicity in tumour cells is enticing increased attention.

Among the various sources of anticancer drugs, microorganisms have more advantages regarding to the potentials in producing diverse compounds and in the manipulation of the production. In this study, we aimed to find a new source of low or non-toxic, natural anticancer agent produced by yeasts, isolated from coastal mangrove ecosystem and used their culture extracts to screen for the cytotoxicity specific to cancer cell lines *viz*. Vero, HepG2 and MCF-7

MATERIALS AND METHOD

Extraction of Yeast crude protein

Cells of S. *cerevisiae* were grown in Yeast Malt Agar medium. After 48 hours of incubation at 24 °C, the cell was harvested by centrifugation and washed twice with 50 mm-Tris–HCl buffer (pH 7.5). Cells were shaken vigorously for 2 hrs at 4°C and then pelleted. The supernatant was concentrated by ultrafiltration using a pore-size of 10,000 Da (Amicon®, Ultra, and Millipore) at 4 °C. The concentrate was stored at −80 °C. Protein quantification was performed using the Bradfor assay (Bradford, 1976), (Bio-Rad, Richmond, CA, USA).

Cell line Culture

Cell lines were obtained from National centre for cell sciences Pune (NCCS). The medium and Trypsin Phosphate Versene Glucose (TPVG) was brought to room temperature by thawing. The tissue culture bottles were observed for growth, cell degeneration, pH and turbidity in inverted microscope. After the cells became 80% confluent sub culturing was done. The mouth of the bottle was wiped off by using spirit soaked cotton to remove the adhering particles.

The growth medium was discarded 4-5 ml of Minimal Essential Media (MEM) was added without Fetal Calf Serum(FCS) and rinsed gently by tilting. The dead cells and excess FCS were washed out, and the medium was discarded. TPVG was added over the cells, incubated at 37º C for 5 minutes for disaggregation. The cells become individual and were present as suspension. 5ml of 10% MEM was added to FCS by using serological pipette. Passaging was formed with serological pipette. After passaging the cells were split into 1:2 and 1:3 ratios for cytotoxicity studies by plating method.

The breast cancer (MCF-7) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), (100U) 20µg/ml penicillin, and 100 µg/ml streptomycin. Incubation was carried out at 37 °C with an atmosphere of 5% CO_2. Normal breast (MCF-7) cells were cultured in 1:1 mixture of DMEM and Ham's F12 medium with 20 mg/ml of epidermal growth factor (EGF), 100 µg/ml cholera toxins, 0.01 mg/ml insulin and 500 µg/ml hydrocortisone, and 5% chelex treated horse serum. Purified berberine and tamoxifen were dissolved in dimethyl sulfoxide (DMSO) and used for the bioassays.

Preparation of cells and Stock drug

After homogenization, 1 ml of suspension was poured in each well of microtitre plate and kept in desiccator under 5% CO_2 atmosphere. After two days of incubation the cells were observed in inverted microscope.(lobomed) magnification(40x)

0.05 ml of drug was dissolved in 4.95 ml of DMSO to get a working concentration of 1 mg/ml. The working concentration was prepared freshly and filtered through 0.45 micron filter before each assay.

In brief, 5 ml of extract was prepared in the concentration of (1mg/ml). 500µl of MEM without FCS was taken in eight Eppendorf tubes for each sample. The samples were syringe filtered using 0.45 µM filter to remove contaminants. Then 500 µl of the working concentration of sample was added to the first eppendorf tube and mixed well then 500 µl of this volume was transferred from first to last tube by serial dilution to obtain the desired concentration of the drug. As a result the volume remains constant, but there was a gradual change in concentration.

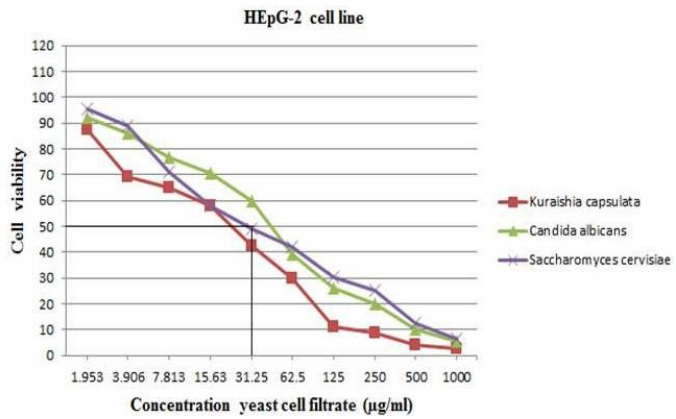

Fig. 1: IC_{50} value of *K. capsulata* ,*C. albicans* , cerevisiae cell -free filtrate on HEP G-2 cell line.

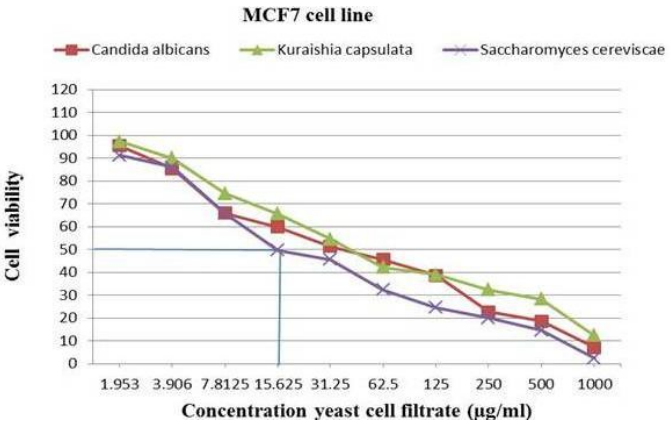

Fig .2: IC_{50} value in *C. albicans* ,*K. capsulate, S. cerevisiae* cell -free S. filtrate on MCF 7 cell line.

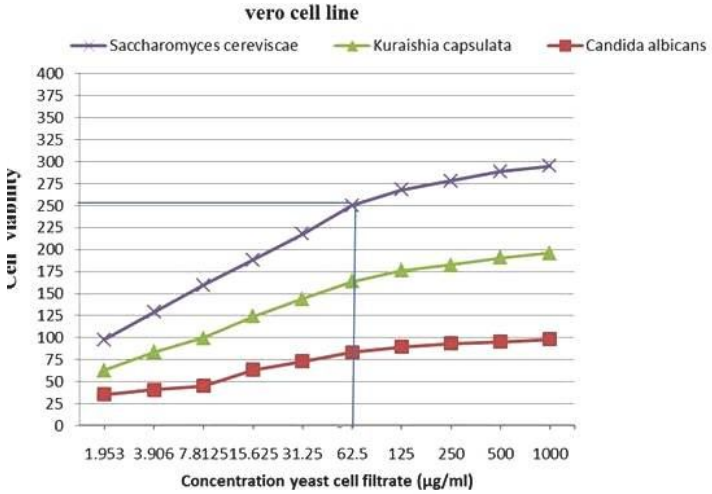

Fig. 3: Effect of the yeast cell -free filltrates at differect concnetrations on vero cell linewith CC_{50} values.

Table 1: $IC_{50,}$ CC_{50} and therapeutic values of three yeasts cell-free extract (CC_{50} is the concentration at which 50% cells survive and IC_{50} is the concentration at which 50% cell death occur).

Yeast	HEP G2 (IC_{50}) (µg/ml	MCF-7 (IC_{50}) (µg/ml)	Normal VERO (CC_{50}) (µg/ml)	Therapeutic index (CC_{50}/ IC_{50})	
				HEP G2	MCF-7
Candida albicans	31.25	31.25	125	2	4
Kuraishia capsulate	31.25	62.5	250	4	8
Saccharomyces cerevisiae	31.25	15.62	250	16	16

MTT Assay

The anticancer activity of samples on MCF7, HepG2 & VERO cells were determined by the MTT (3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide) assay was used to assess the cytotoxicity Horiuchi *et al*.,(1988). Cells (1×10^5/well) were plated in 0.2 ml of medium/well in 96-well plates. For MTT assay the medium from the wells was removed carefully after incubation. Each well was washed with MEM (w/o) FCS for 2-3 times and 200µl of MTT (5mg/ml) was added. The plates were incubated for 6-7 hrs in 5% CO_2 incubator for cytotoxicity. After incubation, 1ml of DMSO (solublizing reagent) was added to each well and mixed well by micropipette and left for 45sec. Presence of viable cells was visualized by the development of purple color due to formation of formazan crystals. The suspension was transferred to the cuvette of a spectrophotometer and the OD (optical density) values were read at 595nm by using DMSO as a blank. Measurements were performed and the concentration required for a 50% inhibition of viability (IC_{50}) was determined graphically Standard Graph was plotted by taking concentration of the drug in X axis and relative cell viability in Y axis.

Cell viability (%) = Mean OD/Control OD x 100%

Results

To evaluate the cytotoxic activity of three different extracts of the marine yeasts isolated from coastal mangrove ecosystem namely *Candida albicans*, *Kuraishia capsulate* and *Sacchromyces cerevisae* against human breast carcinoma cells (MCF7), human hepatocarcinoma (HepG2) and African Green Monkey kidney cell lines (VERO), respectively, were incubated with different doses (1000 to 1.953 µg/ml) of extract. After 24 hours of incubation, cell viability was determined by the MTT assay. Some of the extracts induced cell cytotoxicity in a concentration dependent manner, as illustrated. The results of cytotoxicity assay are presented in (fig .1,2).

All extracts were able to inhibit the proliferation of the cancer cells (MCF-7, HepG2) and the normal Vero cell viability shown (fig .3). The American National Cancer Institute guidelines

(NCI) set the limit of activity for crude extracts at 50% inhibition (IC_{50}) of proliferation of less than 30 mg/ml after an exposure time of 24 h (Suffness and Pezzuto, 1990). IC_{50} values below this stringent point were noted with two extracts at least two of the three studied cancer cell lines, the lowest (31.25mcg/ml) being obtained with sample 1 and sample 2 in human hepatocarcinoma cancer cell lines and 15.625mcg/ml in breast carcinoma cell lines (Table.1). Nevertheless, none of the tested extract was as active as doxorubicin. These data are of interest, as it suggests that the extracts are more toxic to cancer cells than normal cells. Cogitating the overall activity of the extracts, it was exhumed that sample two could be considered as potential anticancer drugs. This is in accordance of the preliminary antitumor studies, which previously demonstrated that extracts showing anticancer effect.

DISCUSSION

Interest in the pharmacological effects of bioactive compounds on cancer treatments and prevention has increased dramatically over the past twenty years . It has been shown to possess numerous anti-cancer activities in various cancer cells through different forms of cytotoxic effects without exhibiting considerable damage to normal cells (Katiyar *et al*., 2009; Mantena *et al*., 2006a). Our observations on toxicity on Vero, HepG2 and MCF-7 cells showed, in agreement with our previous studies (Sassa *et al*., 1987). Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related deaths worldwide, and about half a million individuals die from this disease annually (El-Serag, 2004; Parkin *et al*., 2005). Adriamycin (ADM) has been commonly used as a chemotherapeutic drug in the treatment of HCC (Schwartz, 2004). However, high concentrations of ADM tend to cause severe toxicity to normal tissues, including cardiotoxicity (Bristow *et al*., 1978). Similarly, breast cancer is the second leading cause of cancer-related death in women. Based on American Cancer Society, approximately 200,000 new cases of invasive breast cancer and over 40,000 deaths are expected to occur in 2009 (A.C. Society, 2009). Current anti-estrogen medicine, tamoxifen, is widely used in the prevention and treatment of estrogen receptor positive breast cancer (Lazarus *et al*., 2009). However, a significant number of patients develop tamoxifen resistance and experience severe side effects (Lazarus *et al*., 2009). Thus, it is imperative to search for new alternatives to breast cancer prevention agents. The inhibitory effect of marine yeast on breast cancer cells at different concentrations for 24, 48 and 72 h was studied. Accruing evidences suggest that marine yeast may be a potential chemotherapeutic or a chemopreventive agent based on its ability to induce apoptosis in cancer cells with relatively low toxicity to normal cells. Further studies with *in vivo* and clinical trials needs to be conducted to establish berberine as a safe agent for cancer therapy (Lau *et al.*, 2001).

CONCLUSION

Mangrove-derived yeasts crude extract tested for their anticancer activity. Hence, in the present study, the most interesting biological effect of yeasts the cell proliferation stimulatory activity of very low doses, could be verified also in our recent experiments, using a Vero and two tumor cell lines of human hepatocarcinoma (HepG2), human breast carcinoma (MCF-7). The two yeast species were found to be potent, as evident by low concentration (<63µg/ml) at which 50% of cancer cell death occurred. The IC_{50} value *S. cerevisae* was found more efficient for anti-breast cancer activity.

The yeasts were found more toxic for cancer cells than normal cell line. In this regards,' therapeutic index' is an important parameter to select samples for developing drugs. This value is the ratio of the concentration of the extract at which 50% of the normal cell line survived in normal cell line to that of the extract at which 50% of cancer cell death occurred in cancer cell lines. A drug is considered to be worthy of further testing if it has a therapeutic index value of 16 or greater. The present study found therapeutic index value of 16 in MCF-7 cases of cancer lines for the S. *cerevisae*. This yeast species are promising for its further development as an anticancer drug.

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