IDENTIFICATION OF RADIOPROTECTIVE ACTIVITY IN THE EXTRACT OF INDIAN GREEN MUSSEL, *PERNA VIRIDIS L*

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By

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Dedicated To My Parents

Abstract

The present study describes the radioprotective activity of a hydrolysate prepared from Indian green mussel, *Perna viridis* in terms of dose response survival of *E. coli* and *Saccharomyces cerevisiae*, DNA damage in plasmid and lymphocytes and free radical scavenging activity after exposed to γ-irradiation. The effect of mussel hydrolysate on antioxidant enzymes superoxide dismutase (SOD) and catalase was also studied. The effect of different environmental parameters and biochemical composition of mussel tissue on the biological activity of mussel hydrolysate in terms of infectious activity was also tested in the present study. The mussel hydrolysate (MH), prepared using an enzyme-acid hydrolysis method, was found to increase the survivability of both *E. coli* and *S. cerevisiae* when irradiated in presence of mussel hydrolysate. MH prevented the radiation induced DNA damage and also scavenged the Reactive Oxygen Species (ROS) formation in mice lymphocyte cells. The irradiation of plasmid DNA in presence of MH showed significant protection from γ-radiation induced strand breaks as evaluated by gel electrophoresis. Moreover, the presence of MH during irradiation of isolated mice lymphocytes significantly decreased the DNA damage, as measured by Comet Assay. Measurement of intracellular ROS by dichlorofluorescein fluorescence revealed that the presence of MH effectively scavenged the ROS generated in lymphocytes by both chemical method and γ-irradiation. Further studies revealed that MH enhanced the activity of antioxidant enzymes, superoxide dismutase and catalase in lymphocyte cells. It is concluded that radioprotective activity of the MH was attributable to protection against radiation induced DNA damage, scavenging of reactive free radical species and also by enhancing the activity of antioxidant enzymes, SOD and catalase. The results obtained during present studies further confirmed that neither the influence of environmental parameters nor biochemical composition of the mussel tissue play any significant role in the infectious activity of MH.

CERTIFICATE

This is to certify that the thesis entitled "**Identification of radioprotective activity in the extract of Indian green mussel, *Perna viridis L*"** submitted by **Mr. Sreekumar P. K.** for the award of the degree of **Doctor of Philosophy** in Marine Sciences, Goa University, Goa based on his original studies carried out by him under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any university or institution.

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Place: Dona Paula
Date: 19-10-2007.

All the corrections suggested by Examiners are incorporated.

K.P. Mishra
19.10.2007
Prof. K. P. Mishra
External Examiner

DECLARATION

As required under the University ordinance 0.19.8 (vi), I state that the present thesis entitled **"Identification of radioprotective activity in the extract of Indian green mussel, *Perna viridis L*"** is my original contribution and that the same has not been submitted elsewhere for award of any degree to any other University on any previous occasion. To the best of my knowledge the present study is the first comprehensive work of its kind from the area mentioned.

The literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

(Sreekumar P. K.)

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GENERAL INTRODUCTION

Oceans are extremely rich in living resources and surprisingly though hundreds of million tones of organic materials are produced in the world oceans every year, only a small fraction is recovered as fish and used for human consumption. With a coastline of 7,000 km and an exclusive economic zone of 2.2 million square kilometers, India's total marine fish catch is about 2.4 million tones per year. Among the large variety of organism, many plants and animals possess antibacterial, antifungal chemicals, antifouling, antipredation and others with therapeutic properties (Chatterji *et al.*, 2002). The marine mussels, among mollusks, are one such group of bivalves that produce important chemicals for us (Chatterji *et al.*, 2002).

Mussels belonging to the family mytlidae can easily be identified from other molluscs by the presence of an equilateral shell. These animals are commonly found in the intertidal and subtidal coastal waters up to a depth of 15 m. Mussels being sessile in nature prefer rocky open coasts and are always found attached to the rock pilings and other hard substrates. They secrete byssus threads with which they attach to substrates. Mussels are typical marine animals but can thrive in estuaries where salinity range is between 8 and 20 ppt (Chatterji *et al.*, 1984).

Mussels are distributed in the North Indian Ocean around mainland coast of Southeast Asia, Philippines, South Africa, New Zealand, China and Siam. In India, mussels are found both along the east and west coasts. The distribution of mussels on the east coast is restricted as they occurred only in a small scanty bed in Chilka Lake, along the coasts of Vishakapatnam, Kakinada, Chennai, Pondicherry, Cuddalore, Porto Novo and Port Blair (Kuriakose and Nair, 1976). The abundance of these animals is relatively higher along the west coast – in the coastal waters of Quilon, Alleppey, Cochin, Calicut to Kasargod, Mangalore, Karwar, Goa, Bhatia creek, Malwan, Ratnagiri and Gulf of Kutch.

In India, two species of mussels namely *Perna viridis* (green mussel) and *Perna indica* (brown mussel) are found (Kuriakose and Nair, 1976). They contribute to a sustenance fishery especially along the south west coast of India. Mussel meat is considered to be a cheap source of protein and also as a delicacy in some places like in Goa. Mussels are usually collected from the natural bed and sold in the local market for human consumption. A part of the mussel catch has also been exported (Chatterji, 1984).

Apart from their natural occurrence, mussels can be cultivated in the coastal waters. The technology of cultivation of green mussel on a floating raft in the open sea has already been developed and demonstrated several times in Goa by the National Institute of Oceanography, Goa. The culture of green mussel on floating raft and long lines showed promising results by which a production of 480 tones per hectare per year can be achieved against a very low natural production of 1 tone/year/hectare. The technology for culture of mussels has been transferred to NORAD for its transfer to user communities in rural areas.

Recent studies showed that mussels are not only an inexpensive source of protein for human consumption but also possess some complex bioactive compounds, which have enormous potential in biomedical science (Chatterji *et al.*, 2002). A team of Russian scientist, in 1969, discovered an antiviral compound in the meat of the blue mussel (*Mytilus edulis*), which opened a new chapter in this field (Boikov *et al.*, 1997). An extract prepared from the brown mussel, a sister species of green mussel, has been found to possess both prophylactic and therapeutic properties (Boikov *et al.*, 1997). It can cure viral diseases such as influenza, *Herpes simplex*, *Herpes zooster*, *hepatitis*, *flu* and even *Respiratory Syncytial Virus* (*RSV*). The extract is commonly called as mussel hydrolyzate and sold in the Russian market as a drug. The drug is reported to possess immunomodulatory properties. The Drug and Food controller in Russia has already approved it after all clinical, toxicological and pharmacological tests. It is surprising to know that drug has been proved to be more effective in people living under conditions of high background of nuclear radiation, unfavorable ecological condition and thickly populated areas such as factories, schools, institutes and army divisions (Boikov *et al.*, 1997).

Studies were carried out to understand the efficiency of mussel hydrolyzate. The use of mussel hydrolyzate among children at kindergarten and pre-school centers has been found to reduce incidence of infectious diseases and post infection effects by four times. The Russian government has strongly recommended the use of mussel hydrolyzate for the victims of the Chernobyl disaster. It has been reported that after the use of mussel hydrolyzate, many of the victims showed improvement in health (Boikov *et al.*, 1997). Among children, the use of mussel hydrolyzate for two months has reduced the incidence of flu and acute respiratory infection by five times lower than other children who did not use it (Boikov *et al.*, 1997).

The Russian scientists have also studied the chemical composition of the mussel hydrolyzate. It has important constituents such as albumen (22%), mineral salts (22%), microelements (iodine, copper, silver), macro elements (phosphorous, calcium ferrum), vitamins (B, B2, PP, AE), lipids melononidins and oligopeptides) (Boikov *et al.*, 1997). In the albumen taurine is the most important component. The amino acid component is also very higher as compared to chicken egg, which is considered to possess highest concentration of amino acids so far. Lysine, metionin and tryptophan are the main components present in the mussel hydrolyzate (Boikov *et al.*, 1997). These constituents are considered to be highly nutritional. The other important amino acids present in the hydrolyzate are gistodine, tyrosine and arginine. These amino acids are useful in maintaining human vitality. The polyunsaturated fatty acids with low fatness are also found in mussel hydrolyzate. The microelements reported in mussel hydrolyzate are 10 times higher in concentration as compared to fish and meat. It has also been reported that a gram of fresh mussel meat contains approximately 35 mg of donamin, a substitute to adrenaline, which has tonning up effect on the cardiovascular system in humans. The presence of taurin (2 amino sulfanilic acid) in mussel hydrolyzate is reported to help in proper regulation of heart function, osmotic processes at cellular levels with glycosides intoxication. Taurin has also been found as an effective drug for encephalitic syndrome, cataract and glaucoma. It is also useful as a neuromodulator and neuro inhibitor of the central nervous system, which could effectively be used as an anti-convulsion drug.

Considering the marine mussels as a source of antiviral drugs, attempts have also been made in countries like Japan, Netherlands, Italy, France and New Zealand to identify and isolate some more bioactive compounds from them and other related species. These studies also showed that extract prepared from the bivalves could effectively used to cure the patients suffering from viral diseases and also to prevent spread of viral diseases to others (Chatterji *et al.*, 2002). The scientist of Denmark isolated an anti-rheumatic drug. New Zealanders have prepared a new drug useful for arthritis and nervous problems (Boikov *et al.*, 1997). Russian scientists have isolated mitilan a carbohydrate rich protein complex which provided more body resistance and fighting power not only against the dreaded *Bacillus tuberculosis* bacteria but also against the toxins produced by them (Boikov *et al.*, 1997).

Research done by scientists in various countries and the products made from the marine mussels remained at the laboratory level till 1990 due to lack of clinical and pharmacological data which were required for approval as a drug for human use. However, the Russians accepted the challenge and took a lead to generate these data. They have designed a pilot plant for the extraction of mussel hydrolyzate. As the basic component showing antiviral properties is acid hydrolyzate, the extraction principal based on the acid hydrolyzing process. About fifteen leading medical institutions were involved in this programme. All the tests carried out in these laboratories showed that the hydrolyzate besides having an immunomodulatory effect, it also helped greatly in building resistance in organism against various types of toxins, ultra violet rays and ionizing radiations (Boikov *et al.*, 1997). The mussel hydrolyzate has been found effective in stimulating the restoration of blood production and radiotherapy of tumors. The scientists working at the State Enterprise-Gyprorybflot have designed and developed a new biodigestor for the extraction of the hydrolyzate from blue mussel and subsequently the biodigestor was granted an international patent (No RU 2043109).

It took few years for the scientists to standardize the process of extraction of mussel hydrolyzate. Finally they achieved considerable success in developing a new drug -Viramid from blue mussel (Boikov *et al.*, 1997). The drug was patented as a product. Elaborate studies were carried out at the Pasteur institute, St' Petersburg, Russia which showed that drug was more effective than remantadin used for curing patients suffering from flu (virus strains A_1, A_2 and A). The later drugs are only effective

against flu virus strain A. Viramid has shown its effectiveness on adult patient suffering from herpes I and II viruses with no side effect. Toxicological studies of viramid were completed on the patients suffering from herpes by analyzing urine tests and changes in clinical blood profile. The drug has undergone for further clinical tests at Vishnevsky Surgery Institute, Russia where the scientists have found that the drug was effective in stimulating human immune system and resistance to inflammation. It has also been reported that the drug helped in intensifying the regeneration process in burn wound. On the basis of these results, the drug has been recommended as an anti-burn tonic. Herpes ointment and antiviral toothpaste are the other products made out of the mussel hydrolyzate. It has been confirmed that the use of mussel hydrolyzate by people suffering from acute and chronic virus hepatitis A, B, C and D quickened the recovery.

During the hydrolyzing process for the extraction of mussel hydrolyzate, only a portion was considered useful as drug and rest of the part was discarded. But recently, the Russian scientists have found a protein vitamin- rich compound in the discarded part (by product), which was very useful for animals. This particular substance was reported to be useful in removing toxins from animal's body, providing resistance against different viral strains, enhancing the protein synthesis, stimulating tissue regeneration, enhancing animal growth and normal development (Boikov *et al.,* 1997). The Russian scientists have got a patent on a new drug called midivet, which has been recommended for use in animals. It took almost ten years for the scientists to confirm the use of this drug on animals especially in poultry, pigery and other farm animals and pets like cats and dogs.

Midivet is a tonic and generally given with feed or dissolved in water at the ratio of 1:3. The use of midivet for 7- 10 days has been found effective in the young ones where dosages are repeated after 1-3 months to stimulate the immune system in the animal. This drug has been found more effective for the prevention of many diseases caused by viruses. The studies carried out at poultry and pig farms at St' Petersburg and Moscow, showed promising results. The egg production of chicks has been found to increase by 30 per cent after the treatment with midivet. The survival rate of chickens also increased by 25 per cent, bird grew faster and developed thick fur.

The mussel hydrolyzate extracted from the Indian green mussel was analysed by Indian scientists to identify the presence of antiviral activity at the Pasteur Institute, St' Petersburg, Russia. The experiments have shown encouraging results and antiviral activity observed to be the same as reported in Russian blue mussels. The scope for producing mussel hydrolyzate from the Indian green mussel on a commercial scale is relatively high as the availability of the animal for more than nine months in a year in the natural environment. More over, a technology is available for artificial cultivation of the green mussel under confined conditions in coastal areas.

Ionizing radiation and its damages

Soon after the discovery of X-rays in 1895 by Wilhelm Conrad Rontgen (Rontgen, 1895) and natural radioactivity in 1896, ionizing radiation started for the treatment of cancer. Dr Leopold Freund of Vienna, who successfully treated a benign hairy naevus in 1987, gave first rational application of X-ray therapy. E. H. Grubbe, a physicist at the Hahneman Medical College in Chicago was the first person who treated a patient suffering from breast cancer in the year 1964. But chemical evidences mainly from the effects on the skin, indicated that the ionizing radiation was harmful to human tissues.

Ionizing radiation is basically an electromagnetic wave or a particle capable of producing ions in its passage through the matter and causing immediate chemical alterations in biological tissues. Ionizing radiation damage is caused either by direct interaction with target molecules or indirectly by formation of chemically active free radicals produced mainly by radiolysis of water molecules. Radiation absorbed interacts almost exclusively with electrons of atoms and tissue molecules (Varanda and Tavares, 1998). The resulting molecule contains an unpaired electron in one of its orbital and forms the free radicals.

Due to the predominance of water in the tissues, most of the ionizing events occur in this molecule. Radiolysis of water is reported to generate molecule of hydrogen peroxide (H_2O_2), and a number of highly active radicals such as hydroxyl radical ($OH^{\bullet}$) and superoxide ($O_2^{\bullet}$) (Dragaric and Dragaric, 1971). The radiation damage to a cell has been found to potentiate or mitigate depending on several factors like presence of

oxygen, sulfhydryl compound and other molecules in the cellular milieu (Haynes, 1975). The nuclear region of the cell is 100 times sensitive than cytoplasm and DNA has been reported as the principal cellular target of ionizing radiation (Wiseman and Halliwell, 1996). The damage to DNA includes single strand or double strand breakage which in turn leads to delay in cell division, formation of modified cell, neoplastic formation, point mutation, causing chromosomal aberration, cell loss and ultimately the cell death (Wiseman and Halliwell, 1996). Ionizing radiation also affects other macromolecules like proteins and lipids. Enzyme may lose their active site due to changes in their 3-D structures (Frei, 1994). Cellular membrane is another major target of radiation damage and oxidative attack. Damage to cell membrane or intracellular membranes, has been expressed as an altered permeability resulting in transfer of unwanted molecules from one cellular compartment to another. This has been reported to produce unbalanced metabolism and finally lead to cell death (Stuart and Stannard, 1968). Frei (1994) reported that this is due to lipid per-oxidation of polyunsaturated fatty acid (PUFA) with double bonds, largely present in the phospholipid of membranes. Oxidative damage to lipids has been reported due to lipid per-oxidation which is the autoxidation of the polyunsaturated fatty acid resulting in formation of side chains of lipids by a radical chain reaction. Unchecked per-oxidation decomposition of membrane lipid is reported to be a catastrophic for living system. Lipid peroxidation has been found to cause the formation of malondialdehyde and 4-hydoxy-nonenal which can be reacted with DNA and thus resulting in mutagenic or with protein causing structural and/or functional damages (Frei, 1994).

Importance of radioprotection

In radiation therapy, high-energy beams of radiation are focused on the tumor site from which cancers are removed. Radiation works by causing damage or changes to the cells in the tumor site. The goal of radiotherapy is to give a high enough dose of radiation to the tumor site in order to kill as many cancer cells as possible while still allowing the normal cells to repair and recover. Overtime, this focused radiation damages to cells that are in the path of beam of normal cells as well as cancer cells. As the tumor cells proliferate very rapidly, they usually overgrow their vascular supply resulting in centrally necrotic and hypoxic regions. To prevent tumor formation, higher doses of radiation have been recommended. This is clinically not feasible since the

normal tissues surrounding the tumor are well profused; vascularised and remained oxygenated as such they are more prone to radiation damages (Dave *et al.,* 1991). In order to obtain better tumor control with a higher dose, the normal tissues have to be protected against radiation injury. This necessitates the protection of normal cells surrounding the tumor from radiation injury using an effective radio-protecting compound (Nair *et al.,* 2001). Thus the role of radioprotective compounds is very important in clinical radiotherapy.

Radio protectors are chemicals or modifiers used to prevent radiation damages by a process of ionizing radiation to a living system. The radio protecting substances have shown to reduce mortality when administered to animals prior to exposure to a lethal dose of radiation. This fact is of considerable importance since it permitted the reduction of radiation-induced damages and provided prophylactic treatment against damaging effects produced by radiotherapy. The radio-protecting substances are mainly free radical scavengers and helped in repairing the damages by hydrogen donation to target molecules. These compounds are also helpful in formation of mixed disulfides causing delay in cellular division and induction of hypoxia in the tissues. Radio protecting agents have also been found to minimize the normal tissue injury that is caused by the radiation. The identification of radiation–protecting agents is an important goal for radiation oncologists and basic radiation biologists.

History of radio protectors

The history of radio protectors was started in the year 1900 when Moses Gomberg discovered an unusual carbon compound - triphenylmethyl radical. This finding has opened a new chapter of short-lived radicals as intermediates in several chemical reactors. It was Dale (1942) who termed these compounds as radio-protectors or radio protective agents and carried out several studies using enzymes as indicative molecules. However, Patt *et al.* (1942) for the first time conducted a study, which generated interest in radio protective drugs for human use. In their study cysteine - a sulfur-containing amino acid was administered intravenously to rats 15 minutes prior to the treatment of a lethal dose of radiation. A significant increase of surviving of animals was observed. More efficient radio protective agents were found at later stages, which are generally, sulfur containing compounds.

The research on development of radioprotectors commenced with the Manhattan project in the US and Walter Reed Army Research Institute. The scientists involved in this project have synthesized and screened about 4500 compounds for this purpose. However, only some compounds have shown such characteristic that could be used in protecting the people. Some of the substances under specific circumstances have shown a considerably protective effect on animals. Among these, except one compound "Amifostine" finds applications in radiotherapy of cancer to protect normal tissues during radiation exposure in recent time. None of these compounds has been found suitable for human applications due to acute toxicity (Tannehill *et al.,* 1996). The development of a safe and effective non-toxic radioprotector for human use has remained elusive till today. In fact, no radioprotective agent is now available, either alone or in combination to meet all the requisites of an ideal radioprotector (Maisin *et al.,* 1993). Several compounds which have been found very effective in the laboratory studies, have failed in human applications due to acute toxicity problems, their side effects, or lack of significant protective effects (Turner *et al.,* 2002; Weiss and Landauer, 2003). The protection of healthy tissues during radiotherapy for cancer has been one of the strong motivations for continuing research on exogenous radioprotectors. Experimentation on animals ultimately proved sufficient assurance of biomedical reality and clinical application of such radioactive compounds (Singh *et al.,* 1990).

Classification of radioprotective agents

Radioprotective agents can be classified into three major groups (Nair *et al.,* 2001). They are; 1) chemical radioprotectors; 2) adaptogens; and 3) absorbents.

1) **Chemical radioprotectors**: They are generally sulfhydryl and other antioxidants compounds. This group includes several myelo-; entero- and cerebro-protectors (Livesey and Reed, 1987).

2) **Adaptogens:** Adaptogens have been reported to act as stimulators of radioresistance. These compounds are natural protectors and offered chemical protection under low levels of ionizing radiation (Nair *et al.,* 2001). Generally they are extracted from the cells of plants and animals and showed least toxicity. They can

influence the regulatory system in exposed organism, mobilize the endogenous background of radioresistance, immunity and intensify the overall nonspecific resistance of an organism.

3) **Absorbants:** Absorbants have been reported to protect organisms from the internal radiation and chemicals. These include drugs which prevent the incorporation of radio-iodine by the thyroid gland and the absorption of radionuclids Cs ^{137}, Sr ^{90}, Pu ^{239} etc.

Different types of radio protectors and their mechanism of action

Sulfhydryl radioprotective compounds

Analogues of cysteine and mercaptoethylamine are the early Sulfhydryl compounds tested for radioprotection (Table GI.1). The synthesis of aminoethyl isothiourea (AET) helps us to better understand the structural features of sulfhydryl compounds which are cardinal for radioprotection (Livesey and Reed, 1987). The most effective compounds are those with sulfhydryl groups at one end and 2 or 3-carbon chain - a strong basic amino group at the other end. The synthesis of WR2721 or amifostine or ethiofos [s-2-(3- aminopropylamino) ethylphosphorothioic acid] has been the major breakthrough in the development of radioprotective drugs (Glowe *et al.*, 1984; Weiss, 1997). WR 3689, WR77913, WR 151327, WR638, and WR 44923 are the other important radioprotectors of this series have earlier been reported. All these compounds are water soluble and as such they are easy for administration. Their chemical structures differ with respect to only on the length of the aminoalkyl group, the presence or absence of a methyl group at the terminal end and/ or a hydroxyl group at the alkyl chain. The phosphorylated aminothiols have been reported to be better than other aminothiols with respect to their activity, tolerance and duration of action. However, many of them have been found to show severe side effects, such as nausea, vomiting and hypotension (Maisin *et al.*, 1993; Maisin, 1998).

Among the various sulfhydryl radioprotectors, the one that has undergone a large number of clinical trials and currently in use as an adjuvant in radiotherapy is WR 2721 (Wasserman, 1994; Tannehill and Mehta, 1996). The selective radioprotection of normal cells by this drug has been related to its differential absorption by normal and malignant tissues and its conversion into an active sulfhydryl compound (WR 1065) in normal tissues by alkaline phosphatase action (Travis, 1984; Wasserman, 1994; Valles *et al.*, 1995; Tannehill and Mehta, 1996). The prior treatment of patients undergoing radiation and chemotherapy with this drug has significantly found to reduce hematologic, mucosal and renal toxicity as well as the frequency of neuropathy. It remains one of the most promising compounds at present in clinical radiation therapy for protecting normal tissues because it is safe and easy to administer in a clinical setting. The maximum tolerated single dose of WR 2721 has been reported to be only 740-mg/m^2 body surface when it is administered over a period of 15 min. This drug has been found to provide effective radiation protection when administered immediately prior to radiation exposure (Tannehill and Mehta, 1996). This drug has also been found to reduce the toxicity of a cisplatin treatment in patients with metastatic breast cancer (Ramnath *et al.*, 1997). It has also been found to protect late radiation toxicity to pelvic organs without interfering with beneficial effect of radiation therapy and decrease the hematological and mucosal toxicity (Capizzi and Oster, 1995).

Free radical scavengers and antioxidants

Several aliphatic alcohols including ethanol, ethylene glycol and glycerol are found to be good free radical scavengers. However, these are not suitable for clinical applications because of their toxicity at radioprotecting concentrations. Metodiewa *et al.* (1996) have reported two compounds namely; Tempace and Troxyl- that are 2,2,6,6-tetramethyl piperidine derivatives and their action as scavengers of superoxide inhibitors of iron and ascorbate driven Fenton reaction. These two compounds may prove to be promising antioxidants and radioprotectors in clinical settings depending upon their trials and pharmacological applications.

Melatonin: Melatonin (N-acetyl-5-methyloxytryptamine), a pineal gland hormone involved in regulating the neuroendocrine axis is reported to be highly efficient scavenger of hydroxyl and peroxyl radicals besides peroxynitrite. This is also an important antioxidant compound (Pieri *et al.*, 1994; Pierrefiche and Labroit, 1995; Reiter, 1995; Vijayalaxmi *et al.*, 1996). Melatonin offered significant protection when administered to mice prior to radiation exposure, as assessed by the frequency of chromosomal aberrations in spermatogonia, spermatocytes and micronuclei in bone-marrow cells (Badr *et al.*, 1999). Human peripheral blood lymphocytes that are pre-treated with melatonin showed radioprotection *in vitro* as assessed by the formation of chromosomal aberrations and micronuclei (Vijayalaxmi *et al.*, 1996, 1998). The mechanism of action of this compound has been documented elaborately by the above mentioned authors.

Melatonin in the nucleus is bestowing a direct protection by reducing the extent of primary DNA damage by scavenging the radiation-induced free radicals. The melatonin is also reported to act at the membrane and in the cytosol and generate 'signal(s)' that trigger the activation of one or more of the existing DNA repair enzymes. This activation or set of gene(s) has been found to lead to *de novo* protein synthesis which associated with DNA repair (Vijayalaxmi *et al.*, 1998). In mice high doses of melatonin (e.g. 250 mg/kg body weight) has been found non-toxic and effective in protecting the animal from lethal effects of acute whole body irradiation (Vijayalaxmi *et al.*, 1999). The study of Badr *et al.* (1999) has shown that melatonin administration conferred protection to mice against damage inflicted by radiation when it was given prior to exposure to radiation. Melatonin radioprotection has achieved by its ability as scavenger of free radicals generated by ionizing radiation. Pre-incubation of cultured skin fibroblasts with melatonin showed a significant preventive effect by increasing the absolute number of surviving cells and decreased the level of malonaldehyde (Kim *et al.*, 2001). This shows that melatonin pre-treatment inhibits the radiation-induced apoptosis where melatonin exerts its radioprotective effect in decreasing the lipid peroxidation without involvement of the p53/p21 pathway. Several clinical reports indicate that melatonin administration, either alone or in combination with traditional radiotherapy, results in a very high radioprotecting effects.

Vitamins: Antioxidants like vitamin C and E also offer radiation protection, because radiation damage mimics the oxidative stress associated with active oxygen toxicity (Wilson, 1983). Vitamin C has been reported to protect Allium (Selimbekova, 1969) and barley seeds against radiation (Conger, 1975). The radioprotective effects of ascorbate have been observed to be due to its interactions with radiation-induced free radicals (Duschense *et al.,* 1975). Earlier studies had shown a protection factor of 2 for dietary vitamin C following irradiation from I^{131} (Narra *et al.,* 1993). Ascorbic acid pre-treatment inhibited the radiation-induced elevation in lipid peroxidation and significantly elevated the antioxidant enzymes (Jagettia *et al.,* 2004). It protected mice against radiation-induced sickness, mortality and improved the healing of wounds after exposure to whole body irradiation. It also leads to an early recovery from radiation injury (Mallikarjuna and Jagetia, 2004). The significant inhibition of the biochemical alterations in liver thus suggests the prophylactic role of vitamin C against γ-radiation (Gajawat *et al.,* 2004).

The biologically and chemically most active form of vitamin E is alpha-tocopherol. It is one of the most abundant lipid soluble antioxidants found in humans. Alpha tocopherol is particularly effective as chain breaking antioxidant, thus inhibiting lipid peroxidation. This may play an important role in preventing atherogenic modification of low-density lipoprotein (Frei and Gaziana, 1993). Several studies on the radioprotective effects of vitamin E on normal cells in animals have been established by many workers (Sarma and Kesavan, 1993; Felemoviious *et al.,* 1995; Konopacka *et al.,* 1998; Mutlu *et al.,* 2000). However, Parshad *et al.* (1980) stated that Vitamin E a singlet oxygen scavenger, did not scavenge hydroxyl radicals or hydrogen peroxide. Selenium and vitamin E have been shown to act alone and an additive fashion as radioprotective and chemopreventive agents (Borek *et al.*, 1986). Selenium demonstrates protection by inducing or activating cellular free-radical scavenging systems and subsequently enhances the peroxide breakdown, whereas vitamin E offers protection by complimentary mechanism (Borek *et al.*, 1986). Vitamins A, E and K are lipophilic and their local concentrations in specific cellular compartments might sufficiently be high for protective effect, unlike most of the water-soluble sulfhydral compounds for example cysteamines (Borek *et al.*, 1986).

A water-soluble vitamin E derivative, TMG (Tocopherol Monoglucoside) has been found to be very effective in protecting DNA against γ-radiation *in vitro* and also in mice after oral or intraperitoneal administrations (Nair *et al.*, 2003). TMG has been reported to scavenge free radicals and effectively protect the DNA and membranes against ionizing radiation (Kapoor *et al.*, 2002; Rajagopalan *et al.*, 2002). This compound has been found to have radioprotective effect in mammalian systems and yeast (Singh *et al.*, 2001; Nair *et al.*, 2003; Satyamitra *et al.*, 2003). Administration of TMG by intraperitoneal injection to mice following irradiation showed significant prevention of γ-radiation induced chromosomal aberrations in bone marrow cells (Satyamitra *et al.*, 2003). In a multi organ study using mice exposed to γ-radiation, it has been reported that administration of TMG resulted in preferential protection of cellular DNA in normal tissues such as liver, spleen, blood and bone marrow but not for tumor cells (Nair *et al.*, 2004).

DNA binding ligands

The role of Hoechst 33342 as a radioprotector has been investigated by several workers (Martin *et al.*, 1996; Martin and Anderson, 1999). The radioprotection by this compound is mediated by electron transfer and thus the radioprotective activity reported to improve by the addition of electron-donating substituents to the ligand (Martin *et al.*, 1996). Hoechst 33342 has been found to bind the minor groove of DNA at discrete sites and characterised by 3-4 consecutive AT base pairs. Studies have shown that although maximum protection against radiation-induced strand breaks is at the binding sites, there is also some protection of the intervening DNA. This is due to a global radiation protection resulting in reduction by the bound ligand of transient radiation induced by oxidizing species of DNA (Denison *et al.*, 1992; Martin and Anderson, 1999).

Nitroxides: Nitroxides are class of stable free radical compounds that have been used as biophysical probes in electron paramagnetic resonance spectroscopy. Recent studies on many water-soluble nitroxides have shown the radioprotection in animals when these compounds were administered prior to radiation exposure (Hahn, *et al.*, 1994;1998). Tempol a low molecular weight water-soluble nitroxide is reported to be an effective radioprotector. This compound has shown to permeated the cell membrane freely and acted as a superoxide dismutase mimic (Hahn *et al.*, 1999). Tempol has been reported to provide radioprotection when administered 5-10 minutes prior to whole-body

irradiation Hahn *et al.*, 1998). In tumor bearing cells, it provides protection to normal cells but not the tumor cells. The difference in radioprotection may result from enhanced intratumor bioreduction of Tempol to its non-radioprotective hydroxylamine analogue. Scavenging of free radicals and induction of hypotension and bone marrow hypoxia are thought to be reason for radioprotection (Hahn, *et al.*, 1998; 1999).

Angiotensin converting enzyme inhibitors

Angiotensin Converting Enzyme (ACE) inhibitors and Angiotensin 11 type-1 receptor blockers have been found to be effective in the prophylaxis of radiation-induced lung and renal injury in experimental animals (Ward *et al.*, 1993; Oikawa *et al.*, 1997; Moulder *et al.*, 1998 a & b; Molteni *et al.*, 2000). The various ACE inhibitors investigated for radiation protection included pencillamine, pentoxyfyllin and captopril. Studies on these inhibitors have revealed that the blockage of the angiotensin 11 receptor type 1 which is useful for treating radiation induced renal and lung injuries. A rennin-angiotensin system could fundamentally be involved in the pathogenesis of these injuries (Moulder *et al.*, 1998 a & b; Molteni *et al.*, 2000). Captopril (D-3-mercapto-2-methylpropanoyl-L-proline) has been shown to spare early lung reaction induced by fractionated hemithorax irradiation in rats (Ward *et al.*, 1993). Captopril's therapeutic action has been partly ascribed to the prevention of a radiation-induced increase in the pulmonary arterial pressure resulting in less severe edema in an unirradiated lung. Captopril and angiotensin 11 receptor type-1 blockers protected lung parenchyma from inflammatory response and subsequent fibrosis in irradiated animals. The radioprotective effect of captopril may be related to an inhibition of angiotensin 11 system with combined pharmacological properties such as antioxidation, free-radical scavenging and protease inhibition (Molteni *et al.*, 2000). The use of ACE inhibitory drugs and angiotensin 11 receptor blockers opened new possibilities in radiation therapy with high doses of radiaton-related side effects.

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Cytoprotective agents used in chemotherapy

A number of cytoprotective agents have been developed for the protection of normal cells but not tumor cells from the toxicity and damages associated with chemotherapy and radiotherapy of cancer. Mesna (2-mercaptoethanesulfonic acid), dexrazoxane and amifostine are three of the protective agents approved by the United States, Food and Drug Administration which have potential chemo- and radio-protective activities in cancer treatment (Henseley *et al.*, 1999; Highly *et al.*, 1999; Kleta, 1999; Links and Lewis, 1999; Morais *et al.*, 1999; Khojasteh *et al.*, 2000). Mesna has been reported to decrease the incidence of chemotherapy-induced urothelial toxicity in cancer patients. Dexrazoxane has been useful as an adjuvant in the doxorubicin-based chemotherapy of tumors (Henseley *et al.*, 1999, Links and Lewis, 1999, Morais *et al.*, 1999).

Metalloelements and Metallothionin

Metallothionin is a low molecular weight protein of 60 aminoacids containing one third of cysteine and has been shown to protect animals and cells exposed to ionizing radiation (Bakka *et al.*, 1982; Matsubara *et al.*, 1987; Kagi and Schaffer, 1988; Renan and Dowman, 1989; Satoh *et al.*, 1989; Murata *et al.*, 1995; Miko *et al.*, 1998). The administration of metalloelements to animals has been found to increase the synthesis of the protein in various tissues, which involve in the regulation of metabolism of metalloelements, the detoxification of excess metalloelements and scavenging of free radicals (Matsubara *et al.*, 1987). The oral administration of bismuth subnitrate to mice is found to reduce the radiation's lethal effects and bone marrow injury. This radiation protection has been attributed to an induced synthesis of metallothionin in bone-marrow cells (Satoh *et al.*, 1989). The pre-treatment of mice with manganese chloride and cadmium salts has been reported to increase the level of metallothionin in various tissues of the animal and subsequently reduce the lethal effects of whole-body irradiation (Matsubara *et al.*, 1987). Tungstate, vanadate and molybdate salts have insulin-like effects because they increase the basal fructose-2, 6-bisphosphate levels. It counteracts the effects of glucagons and fructose-2, 6-bisphosphate concentrations and 6-phosphofructo-2-kinase activity and also stimulates the glycolytic flux (Fillat *et al.*,

1992). These salts also stimulate adenyl cyclase activity (Hwang and Ryan, 1981). Studies have shown that these compounds at low nontoxic levels protected experimental animals from lethal effects of ionizing radiation through their effect on the hemopoietic system (Zaporowska and Wasilewski, 1992; Sato *et al.*, 1999).

Cytokines and immunomodulators

Various immunomodulators in combination with radiotherapy or chemotherapy have been reported to control tumor growth in experimental animals as well as in clinics. In a randomized clinical study it has been demonstrated that the administration of recombinant interferon gamma resulted in immune stimulation in patients and showed complete remission of cancerous cells after radiotherapy and chemotherapy (Pujol *et al.*, 1993).

Protein associated polysaccharides, such as AM5 and AM218 have been reported to kill *Lactobacillus* cells (Landauer *et al.*, 1997). These compounds have immunomodulating properties and shown to have protective action against radiation. The bacterial extract (Broncho-Vaxom), when administered in combination with indomethacin, an inhibitor of prostaglandin production, to mice prior to lethal irradiation, exerted an additional radioprotective effect (Landauer *et al.,* 1997).

Ammonium trichloro (dioxyethylene-0, 0') telluride (AS101) is a synthetic compound and reported to exhibit immunomodulating properties. It has shown a radioprotective effect on the hemopoiesis upon irradiated of mice and mice treated with various chemotherapeutic drugs. These studies have revealed that the administration of AS101 elevated levels of serum amyloid A (SAA) in the sera of treated mice. It has been shown that interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-alpha) are the important mediators of changes in SAA concentrations induced by AS101 (Kalechman *et al.*, 1995 a & b). The cytokines IL-1, IL-6 and TNF-alpha and the stem cell factor have shown to abrogated the ability of AS101 and increased the survival of lethally irradiated mice (Kalechman *et al.*, 1995 a, b). Various immunomodulating substances have also been reported to afford radioprotection to mammalian organisms (Weiss and Landauer, 1988).

Lipopolysaccharide and prostaglandins

Lipopolysaccharide has been shown to protect the intestine and bone marrow from radiation injury after whole body radiation exposure in mice (Riehl *et al.*, 2000). Studies with cyclo-oxygenase–2 inhibitors and mutant strains of mice having a defective gene for cyclooxygenase-2, revealed that the lipopolysaccharide offered radioprotection in mice through a prostaglandin dependent pathway (Riehl *et al.*, 2000). Prostaglandin and OK-432 have protected mice against radiation injury (Neta, 1988, Joshima *et al.*, 1992). Prostglandins have been found to offer radioprotection to several tissues including gut, bone marrow, hair follicle and male germinal epithelium (Walden *et al.*, 1987; Hanson *et al.*, 1988). Hanson *et al* (1995) have reported the radioprotective action of misoprostol, a prostaglandin E1 analogue. It has been found that this compound selectively protected normal cells from radiation injury while sparing tumor cells (Van Bull *et al.,* 1999). Recent studies on misoprostol with DNA repair proficient and DNA repair deficient cell lines indicated that the radioprotection property is dependant on cell cycle. Additionally, DNA repair could be facilitated by this compound (Van Bull *et al.,* 1999). The induction of a radioresistant state has also been found to be one of the reasons for radioprotection by isoproterenol which normally elevated the cellular cyclic AMP levels. Similarly, cyclic nucleotides have been found to alter the cellular radiosensitivity (Nair *et al.,* 2001).

Plant extracts and isolated compounds

Plant extracts in certain cases have been proved to be very effective radioprotectors. These extracts originate from following diverse group of plants:

Citrus plants: Fruits and leaves of citrus plants are reported to be rich sources of radioprotective compounds. Its flavonoid, hesperdin has exhibited strong antioxidant activity (Miyake *et al.,* 1975). It has also been found that this compound reduced the frequencies of micronucleated polychromatic, normochromatic erythrocytes and protected mouse bone marrow by a factor of 2.2 against the side effects of γ-irradiation (Hosseinimehr *et al.,* 2003).

Hippophae rhamnoides: This plant has antioxidant, anti-inflammatory, antimicrobial and immunostimulatory properties (Goel *et al.*, 2002). The aqueous extract of this plant has been found to enhance the survival of mice when administered 30 minutes prior to whole body irradiation (Goel *et al.*, 2002). Administration of a hydroethanol (50:50 v/v) extract 30 minutes before irradiation increased the number of surviving crypts in the jejunum by a factor of 2.02 and villi cellularity by 2.5 fold (Goel *et al.*, 2003).

Mentha piperita: Oral administration of 1g/kg body weight/day before radiation has been reported to protect against radiaion induced chromosomal damage in bone marrow of mice with a dose-modifying factor (DRF) value of 1.78 (Samarth and Kumar, 2003). Mentha extract and its oil showed enhancement in the survival of mice besides improving hematological parameters (Samarth *et al.*, 2004).

Ocimum sanctum: Uma Devi and Ganasoundari (1995) have reported the radioprotective activity of *Ocimum* for the first time. Aqueous and alcoholic extracts of leaves of the plant have shown radioprotective properties but the aqueous extract (optimum dose: 50 mg/kg body weight; acute LD50: 6 g/kg body weight) is found more effective for the survival of mice. Two active components of the extract, orientin and vicenin reported to increase the survival of mice when administered 30 minutes prior to lethal whole body γ-irradiation. Vicenin has DMF value of 1.37 whereas orientin has 1.30 in the murine system (Uma Devi *et al.*, 1999). These compounds have found to inhibit significantly the Fenton reaction induced by OH radical under *in vitro* conditions (Uma Devi *et al.*, 2000) and protected human lymphocyte chromosomes against radiation (Uma Devi *et al.*, 2001).

Podophyllum hexandrum (Himalayan Mayapple): *Podophyllum hexandrum* has been reported to protect the plasmid pBR322 DNA against radiation-induced damage *in vitro* (Chaudhary *et al.*, 2004). It has been found to enhance the survival of mice and simultaneously increase the levels of liver GST and SOD besides intestinal SOD (Mittal *et al.*, 2001). It has also been observed that it prevented the radiation induced neuronal damage in postnatal rats exposed *in utero* (Sajikumar and Goel, 2003). The extract also showed radioprotection in *Saccharomyces cerevisiae* (Bala and Goel, 2004).

Tinospora cordifolia (Guduchi): The aqueous extract of this plant has been found to enhance the survival of mice against a sublethal dose of gamma radiation injury and help to regain the weight lost. It has also been found to reduce radiation-induced damage in the liver cells (Goel *et al.*, 2004).

Caffeine: Caffeine is reported to scavenge hydroxyl radicals and compete with oxygen for radiation-induced electrons (Singh and Kesvan, 1990; Devasagayam *et al.*, 1996; Kesavan, 2005). Caffeine offers radioprotection against oxygen-dependent radiation-induced damage in barley seeds (Kesavan and Ahmad, 1974), in Chinese hamster ovary cells (Kesavan *et al.*, 1985), rat liver mitochondria (Kamat *et al.*, 1999; 2000) and plasmid DNA (Santhosh Kumar *et al.*, 2001). Caffeine has been reported to be an effective radioprotector in bone marrow chromosomes of mice when given before or after whole body γ-radiation (Farooqi and Kesavan, 1992). Caffeine showed to restore normal cell cycle following X-ray induced arrest in G2 phase in one and two cell mouse embryos (Grinfeld and Jacquest, 1988). Caffeine has also been reported to provide protection against radiation-induced lethality in mice (George *et al.*, 1999).

Chlorophyllin: Chlorophyllin is a semi-synthetic mixture of water soluble sodium copper salts derived from chlorophyll. It has been reported to act as an antimutagen (Ong *et al.*, 1986) and as a radioprotector (Morales *et al.*, 1984; Zimmering *et al.*, 1990; Abraham *et al.*, 1994; Morales *et al.*, 1994; Pimentel *et al.*, 1999; Boloor *et al.*, 2000; Kamat *et al.*, 2000). Chlorophyllin is also considered as a protecting agent of mitochondrial membranes against γ-radiation *in vitro* (Boloor *et al.*, 2000; Kamat *et al.*, 2000), strand breaks and plasmid DNA (Santhosh Kumar *et al.*, 1999), sister chromosomal exchange (SCE) in murine bone marrow cells, *in vivo* (Morales *et al.*, 1984). It has been found to reduce significantly the incidence of micronucleated polychromatic erythrocyte in bone marrow cells upon γ-ray exposure (Abraham *et al.*, 1994). Chlorophyllin also found to exhibit radioprotective activity in *Drosophylla melanogaster* (Zimmering *et al.*, 1990).

Ferulic acid: It is a monophenolic phenylpropanoid occurring in plant products such as rice, green tea and coffee beans. It has shown the ability to act as an antioxidant against peroxyl radical induced oxidation in neuronal culture and synaptosomal membranes (Kanski *et al.*, 2002). The compound has been found to scavenge the reactive oxygen species such as hydroxyl radical (OH), hypochlorous acid (HOCl) and peroxyl radical

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(RO_2) (Scott *et al.,* 1993) besides the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Kikuzaki *et al.,* 2003). Administration of ferulic acid 1 hour prior to radiation significantly reduced the DNA damages in mouse blood leukocyte and bone marrow cells. Ferulic acid given prior and/or immediately after γ-radiation exposure significantly reduced the micronucleated reticulocytes (MNRETs) in mouse peripheral blood leukocytes (Maurya *et al.,* 2005).

Glutathione (GSH): Glutathione is a very important cellular antioxidant. GSH has been reported as a radioprotector of cells in culture (Saunders *et al.,* 1991) and animal *in vivo* (Grozdov, 1987). Patients with higher GSH levels, treated with radiation for squamous cell carcinoma of the oral cavity, showed less severe mucositis (Bhattathiri *et al.,* 1994).

Glycyrrhizic acid (GZA): Root extracts of the plant *Glycyrrhizia glabra* have been reported to have immunomodulating properties (Kores *et al.,* 1997) and antioxidant effects (Vaya *et al.,* 1997). The radioprotective effect of the extract on γ-radiation has been found to induce DNA and membrane damages (Shetty *et al.,* 2002). GZA offered protection to plasmid DNA, pBR322 DNA from radiation induced strand breaks with a dose-reduction factor of 2.04 at 2.5 mM concentrations. Under *ex vivo* condition, GZA protected the cellular DNA of human peripheral blood leukocytes when exposed to gamma radiation in a concentration dependent manner (Gandhi *et al.,* 2004). Pulse radiolysis studies indicated that GZA offered radioprotection by scavenging free radicals (Gandhi *et al.,* 2004).

Troxerutin: Troxerutin, a derivative of the natural flavonoid rutin extracted from plant *Sophora japonica*, scavenges oxygen-derived free radicals (Wenisch, 2001; Kessler *et al.,* 2002). It has also been shown that during radiotherapy of head and neck cancers, administration of a mixture of troxerutin and coumarin offered protection to a salivary gland and mucosa (Grotz *et al.,* 1999). Troxerutin showed considerable inhibition of the lipid peroxidation in membrane of sub-cellular organelles as well as normal tissues of tumor bearing mice exposed to γ-radiation. It has been found that administration of troxerutin resulted in differential protection of DNA in blood leukocytes and bone marrow cells (Maurya *et al.,* 2004). Maurya *et al.* (2005) have also demonstrated the enhancement in the process of DNA repair with concentration dependent radioprotection

to mouse blood and bone marrow cells. They found a significant inhibition of the micronuclei in human peripheral blood lymphocytes and mouse reticulocytes.

Vanillin: Vanillin (4-hydroxy-3-methoxybenzaldehyde) is another antioxidant in lipid assay system reported by Aruoma *et al.,* (1990). Vanillin and its analogs have shown antimutagenic or antigenotoxic effects (Ohta *et al.,* 1988; Shaughnessy *et al.,* 2001). Oral treatment of mice with 500 mg/kg body weight of vanillin on 7.5 hr after the injection of mitomycin C, showed 50% decrease in the frequency of micronucleated polychromatic erythrocytes (Inoue *et al.,* 1988). The antimutagenic effects of vanillin could be the result of a mutation-dependent error free pathway for post-replication DNA repair (Ohta *et al.,* 1988). This compound has been found to suppress the chromosomal aberrations induced by X-rays in V79 cells *in vitro* and in mice on *in vivo* studies (Sasaki *et al.,* 1990). It also showed the inhibition of lipid peroxidation in rat liver mitochondria and reduction in DNA damage in plasmid pBR322 (Kamat *et al.,* 2000; Santhosh Kumar *et al.*, 2000).

Mechanisms of radioprotection

Mechanism that describes the characteristics of radioprotectors is a complex phenomenon and it has still not been well explained. However, it is known that radioprotective activity is accomplished through different mechanism on three special levels of cell organization. In general it includes at molecular, physiological-biochemical and organic levels.

A: Molecular level: This involves; cleansing of free radicals or scavenging of free radical, donation of H atom, bounding to critical biological targets, forming mixed disulfides, delay of cellular division and induction of hypoxia in the tissues.

B: Physiological-biochemical level: The physiological-biochemical level includes hypoxsy, loosening unproteined disulphide, biochemical stroke and hypothermia

C: Organic level: This includes only the stimulating reparation of cells.

A. 1: Cleansing of free radicals or scavenging of free radical

The mechanism of free radical scavenger suggested that certain agents are oxidized by free radicals and form stable compounds making free radicals incapable of reacting with cell vital components. This mechanism prevents the free radicals from reacting with the cell vital components (Varanda and Tavares, 1998). Sulfhydryl compounds like cysteine, cysteamine, glutatathione, WR 2721, other WR compounds, vitamins A, and E render radioprotective mechanism by acting as a free radical scavenger (Nair *et al.,* 2001). Several free radical scavengers are known to interact with aqueous free radicals which in turn prevent the radiation induced lethality of cells. The Radioprotectors may also react with water radicals or radicals of bio molecules (**X**˙) by donating hydrogen atoms to repair the radical species as shown below:

$$2\,X^{\cdot} + 2\,RSH \Rightarrow 2XH + RSSR$$

$$2\,RSH + 2OH \Rightarrow RSSR + 2H_2O$$

A.2: Donation of H atom

Another mechanism that has been demonstrated with polymers is the repair by hydrogen donation. If a R-H molecule is converted into an $R^{\bullet}$ (radical $R^{\bullet}$) by exposure to radiation, a protective agent can donate a hydrogen atom to this radical and subsequently restoring it to its original state (Nair *et al.,* 2001).

A.3: Bounding to critical biological targets and forming mixed disulfides

Radioprotectors can also interact with cellular targets like DNA forming mixed disulfides and prevent radiation damage by stabilizing the target. Several amino thiol radioprotectors, such as cysteamine, guanidoethyl disulfide and glutathione disulfide bind to DNA and their DNA binding capability is parallel to their radioprotective potency (Nair *et al.,* 2001). Sulfhydryl compounds of the aminothiols form mixed disulfides with sulfhydryl compounds of cellular proteins. When one of these disulfides is attacked by free radicals, one of the sulfur atoms is reduced and the other oxidized. However, in this process, the protein has not been found to damage. Therefore, the cellular proteins are protected in 50% of the cases. This theory considers oxidation of sulfhydryl compounds

of cellular proteins as the main factor for occurrence of radiation-induced damage (Varanda *et al.*, 1998). Binding of aminothiols and disulfides to DNA has been considered a potentially important factor in radioprotection. In view of these considerations, Brown *et al.* (1967) proposed that the sulfhydryl compounds of the radioprotective aminothiols acted by binding to DNA and thereby reversibly inhibited replication. They further stabilized their structure which provided additional time for repair.

The radioprotective activity of a number of thiol compounds (RSH, R' SH etc) has been correlated with ratio and extent of mixed disulfide (RSSR') formation (Eldjarn and Phil, 1958). The regeneration of native proteins can be achieved by a thio-disulfide exchange with glutathione, which possibly catalyzed by thiol transferase. Subsequent action of glutathione redox system is coupled with glutathione reductase and NADPH and can be represented as follows:

$$\text{Protein-S-S-R} \Rightarrow \text{Protein- S+ }^{\bullet}\text{SR}$$

$$\text{RSH+R'SSR'} \Rightarrow \text{RSSR' + R'-SH}$$

$$\text{RSSR' + RSH} \Rightarrow \text{RSSR+R' SH}$$

The mixed disulphide hypothesis is limited to the protection of enzymes and proteins. It is difficult to explain the radioprotection of nucleic acids because the SH group is restricted only to proteins.

A. 4: DNA repair and cell recovery process

In several studies, the cellular recovery or repair processes have been found to be enhanced by radioprotectors (Orr, 1984). DNA lesions caused by ionizing radiation could lead to cell death, mutageneciity or altered function, or even cells may recover from radiation-induced effects (Orr, 1984). Endogenous radioprotective substances have been investigated with respect to their role in cellular recovery from radiation and chemical onslaughts (Bump *et al.*, 1981; 1982; Berstsche and Schorn, 1986; Roizin *et al.*, 1984). Thiols, such as glutathione has been reported to be responsible for the repair of DNA single strand breaks. Cells in which GSH deficiency is produced by dl-Buthionine-sulfoxime or by hypoxia or by misonidazole has shown lack of DNA single-strand break repair (Edgreen *et al.*, 1981; Edgreen, 1983; Clark *et al.*, 1984; Revesz and

Edgreen, 1984; Revez, 1985). Drugs and chemicals which stimulate or increase the activity of DNA precursor-synthesizing enzymes such as ribonucleotide reductase etc are also considered as radioprotectors. The administration of the drugs such as indralin and indometaphen prior to radiation exposure to animals (mice and dogs) resulted in higher survival of animals from lethal doses of gamma-radiation (Pulatova *et al.*, 1999). An increase in the activity of the enzyme like ribonucleotide reductase has been reported to occur in several organs of these treated animals. The extent of DNA repair increased as a result of the activation of the DNA precursor-synthesizing enzymes. The stimulated synthesis of deoxyribonucleotides as well as DNA and protein synthesis in irradiated animals has been reported to be the mechanism underlying radioprotection by these drugs (Pulatova *et al.*, 1999).

A.5: Delay of cellular division and induction of hypoxia in the tissues

Oxidation of the thiols has been reported to consume enough oxygen to reduce its tension. It has already been demonstrated that hypoxia also helped in radioprotective activity. Biogenic amines, histamine, serotonin, nor-epinephrine and epinephrine have also reported to manifest their radioprotective action by inducing hypoxia. The induction of hypoxiais considered to be a contribution of the thiols to radioprotection under certain conditions. However, other mechanism might be involved since some compounds exert radioprotective activity without altering oxygen tension on the tissues. There is an evidence of the existence of more than one radioprotective mechanism of a certain compounds and one of the compounds might be more or less important depending on the irradiated system and on the specific radiation conditions (Varanda and Takahashi, 1993).

In addition to these, other radioprotective mechanisms have been reported although little is known about their characteristics. One of these mechanisms is the protection by substitution in which the protective agents work as spare parts and capable of substituting cellular constituents if the biochemical functions are irreversibly damaged (Biaglow, 1987; Sarma and Kesavan, 1993). Other mechanisms are namely; protection by alteration of cellular metabolism, stress caused by irradiation or by other means that lead to an enhancement of radio resistance and castration (Betz, 1955). Modification of ionizing radiation-induced damage in mammals has extensively been studied either from the therapeutic or the prophylactic viewpoints. Experimentally, the most successful

therapy is transplantation of hematopoietic tissue after the irradiation (Smith and Congdon, 1960; Van Bekkum and De Vries, 1967). Prophylaxis can be made by the administration of protective agents prior to irradiation (Bacq, 1965). These agents are either chemical protector such as cysteine and 2-aminoethylisothio-uronium bromide hydro bromide (AET). They have also been found to either reduce radiation-induced damage or enhance regeneration of hematopoietic tissue.

Table GI.1: **Different radio protectors and their mechanism of action**

RADIOPROTECTORS	MECHANISM OF ACTION
Sulfhydryl compounds Cysteine, Cysteamine, Glutathione, AET, WR 2127 and others WR compounds.	Free radical scavenging, donation of H atom
Antioxidants Tempace, Hoechst 33342,Vitamin A, E and C, TMG, Melatonin, etc	Free radical scavenging
ACE inhibitors Captopril, Elanopril, Pencillamine, Pentoxyfylline, L-158, 809 etc.	Protease inhibition (re- ninangiotensin System), antioxidation collagen synthesis inhibition
Cytoprotective agents Mesna, Dexrazoxane, Amifostin (WR 2127)	Reduced toxicity of chemotherapeutic drugs, decrease of urotherial toxicity and nephrotoxicity.
Metalloelements Manganese chloride, Cadmium salts, Bismuth Subnitrate, etc.	Metallothionine induction
Immunomodulators Gamma- interferon, Polysaccharides, AM5, AM218, Heat killed Lactobacillus cells. Broncho-Vaxom, trehalose dicorynomycolate, AS101.	Immune stimulation, increased production of cytokines
Lipopolysaccharides and prostaglandins.	Prostaglandin synthesis, elevated level of cyclic AMP, DNA repair
Plant extracts and isolated compounds Curcumin, Orientin, Vicinin, Caffeine	Free radical scavenging, anti-oxidation
DNA binding ligands Hoechst 33342	Electron transfer, free radical scavenging
Other compounds Melatonin, Carnosin, Tempace, Tempol	Free-radical scavenging, antioxidant, free radical scavenging

CHAPTER 1

Process of preparation of extract from the Indian Green Mussel

The biodigestor

A specially designed biodigestor consisting of two main vessels was fabricated from a local vendor (Plate 1.1). A 5 liter capacity vessel fitted with a geared motor was used as a fermenter in which a constant temperature was maintained with a thematically controlled stainless steel heater. The motor was provided with an impellor to always churn the mixture of the vessel properly. The panel of the biodigestor was also provided with four peristaltic pumps. These pumps were used for transferring reagents and biological materials from one vessel to another. The other vessel was a double layered stainless steel bio-reactor coated with acid resistant enamel polish on both the surfaces. An oil bath with a thermostatically controlled titanium heater was used to maintain a constant temperature of the vessel. This was done by circulating the heated oil of required temperature with the help of a specially designed pump. The pH of the resultant extract was adjusted by adding alkali or acid with the help of peristaltic pumps.

Deshelling of live green mussel

Samples of live green mussels (*Perna viridis* L*)* measuring in shell length of 50 to 65 mm were collected directly from the sea. Mussels were brought to the laboratory, cleaned and de-shelled with the help of a sharp knife. Mussel meat and mantle fluid was removed carefully in the laboratory.

Fermentation of the mussel meat

The mussel meat with mantle fluid was taken in fermenter vessel of the biodigestor to which an enzyme such as (protosubtiline: 10% of the weight of mussel meat) and 6% distilled water was added. The resultant solution was stirred for 2 hours at a constant temperature of 40° C.

Digestion and distillation of fermented solution

The resultant solution in the form of thick paste was transferred into double layered bioreactor for digestion. Concentrated hydrochloric acid (12% of the total mussel weight) was slowly added and then the temperature of the biodigestor was raised to 100± 1° C. The process of digestion was completed in 20 hours.

Cooling of the solution

After completion of the digestion and distillation process, the resultant solution was allowed to cool to room temperature for 10-12 hours.

Neutralization of the solution

The solution was neutralized with sodium hydroxide to achieve a pH of 5.6 by adding alkali with the help of peristaltic pump.

Isolation of active compound

The active extract was isolated from the rest of the solution by keeping the resultant solution in a separating flask for 15 days and carefully removing the middle part of the solution after the formation of three different layers.

Fractionation scheme

The crude extract was cooled in a deep freezer to –80° C and lypholized at –20° C (~10 hours) using a table top freeze dryer (Edwards : Micro Modulo). The lypholized material was dissolved in methanol with stirring using a magnetic stirrer (3-4 hours). The solution was then allowed to settle and then filtered using an ordinary filter paper to yield clear methanol solution and a residue. The process was repeated twice to separate all the methanol soluble part, completely. The residue was dissolved in double distilled water. Both the methanol soluble and water-soluble parts were then freeze dried and kept at 4° C till further use. Biological activities of each fraction were screened to identify the most active fraction.

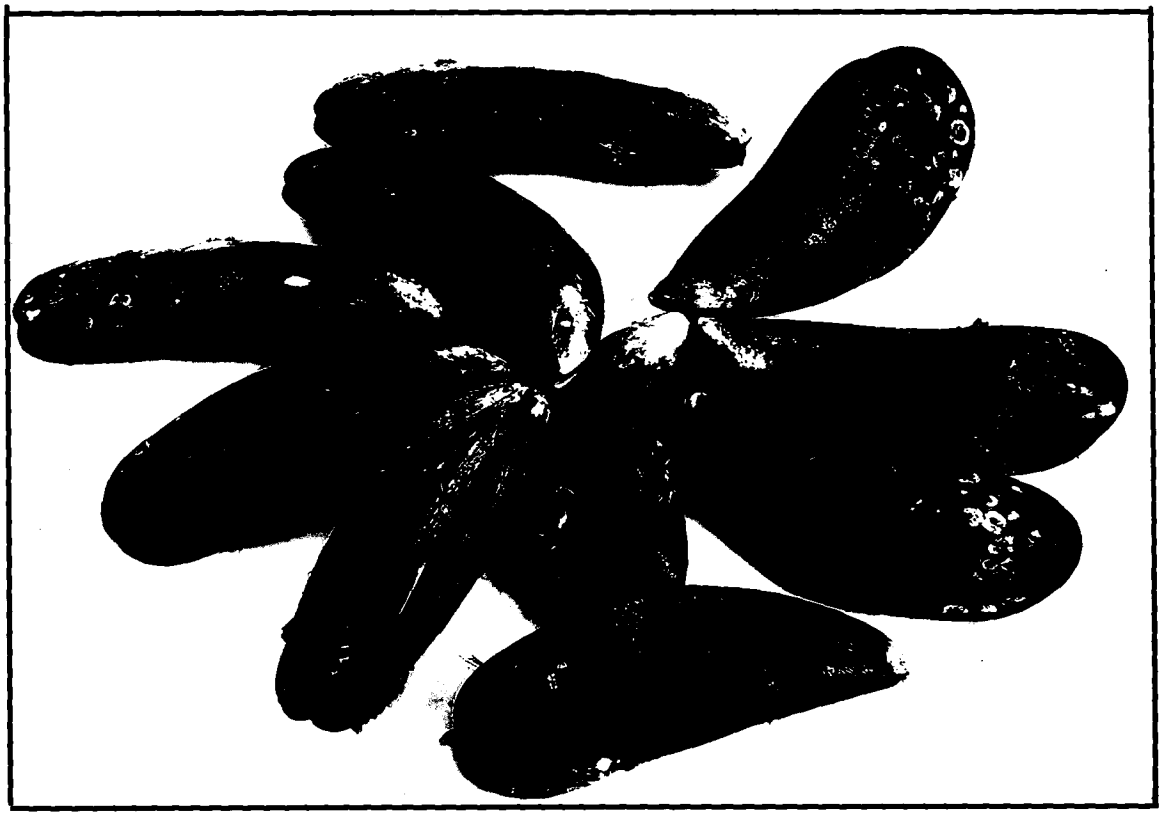


Plate 1 A. Indian Green Mussel, *Perna viridis*

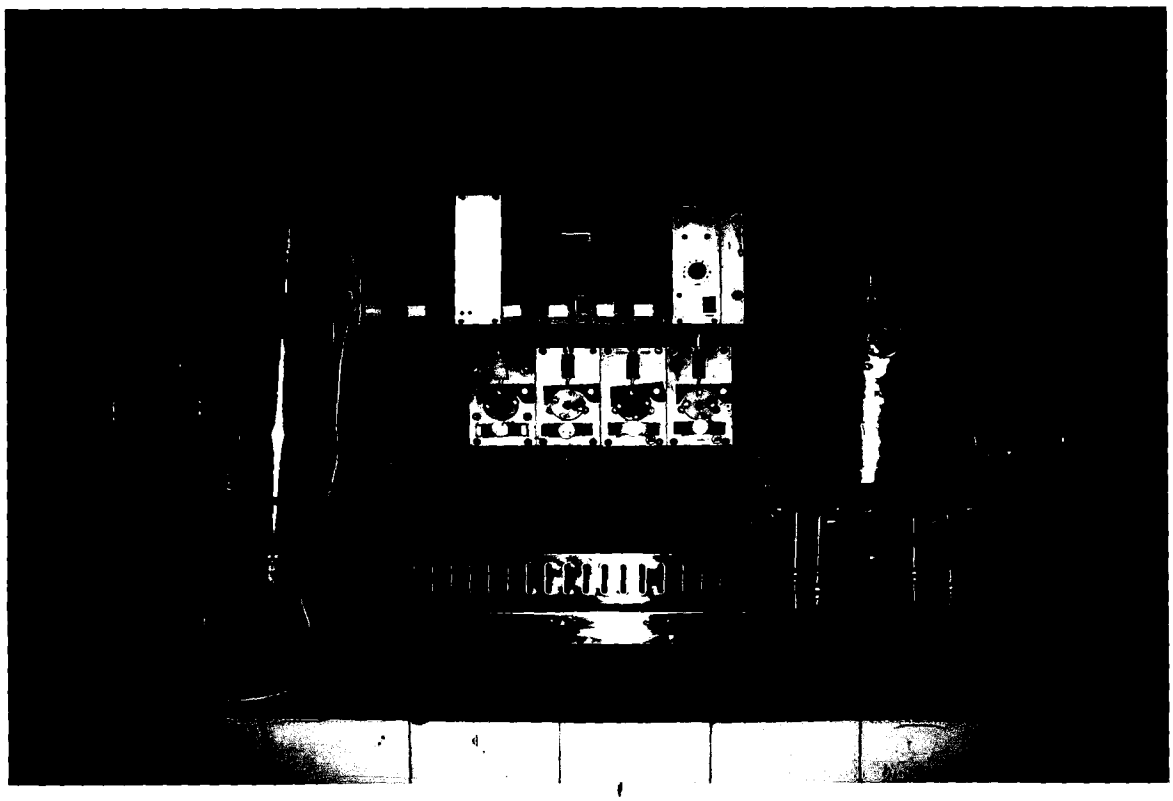


Plate 1 B. Biodigestor used for the preparation of mussel extract

CHAPTER 2

Radioprotective activity of mussel hydrolysate evaluated on *Escherichia coli*

Escherichia coli (*E. coli*) is the largest bacterial groups among *enterobacteriaceae* family, which are facultatively anaerobic *gram-negative* rods live in the intestinal tracts of animals. Medically *enterobacteriaceae* are the most important bacteria. A number of genera within the family are human intestinal pathogens (e.g. *Salmonella, Shigella* and *Yesinia*). Several others are normal colonies of the human gastrointestinal tract (e.g. *E. coli, Enterobacter and Klebsiella*). However, these bacteria, as well may occasionally be associated with disease of humans. *Escherichia coli*, usually abbreviated to *E. coli*, was discovered by Theodor Escherich in 1885, who was a pediatrician and bacteriologist. It is one of the main species of bacteria that live in the lower intestines of warm-blooded animals including birds and mammals. *E. coli*, whipping boy of microbiology and genetics laboratories around the world, is the most encountered bacterium in the clinical laboratory. They are necessary for the proper digestion of food. The presence of this species in groundwater is a common indicator of fecal contamination.

Physiologically, *E. coli* is versatile and well adapted to its characteristic habitats. It can grow in media with glucose as the sole organic constituent. Wild type *E. coli* has no growth factor requirements and metabolically it can transform glucose into macromolecular components making up the cell. Bacterium can grow in presence or absence of O_2. Under anaerobic conditions, it grows by means of fermentation, producing characteristic "mixed acids and gas" as the end products. However, it can also grow by means of anaerobic respiration, since they are able to utilize NO_3, NO_2 or fumarate as final electron acceptors for respiratory electron transport processes. This helps the *E. coli* to adapt well in intestinal (anaerobic) and its extra intestinal (aerobic or anaerobic) habitats. *E. coli* can respond to environmental signals such as chemicals, pH, temperature, osmolarity etc., in a number of very remarkable ways. For example, it can sense the presence or absence of chemicals and gases in its environment and swim towards or away from them. Or it can stop swimming and grow fimbriae that will specifically attach to a cell or surface receptor.

In response to change in temperature and osmolarity, it can vary the pore diameter of its outer membrane poring to accommodate larger molecules (nutrients) or to exclude inhibitory substances. With its complex mechanisms for regulation of metabolism, the bacterium can survey the chemical contents in its environment and synthesizes the required enzymes, well in advance. The bacteria do not wastefully produce enzymes for degradation of carbon sources unless they are available. Similarly, the bacteria do not produce enzymes for synthesis of metabolites if they are available as nutrients in the environment. The bacterium popularity boomed in the 1940s when scientists figured out how to use it to pry open the secrets of genes. In 1970s scientists figured out how to insert foreign DNA into *E. coli* turning them into biochemical factories that could chum out valuable compounds such as insulin. Research in *E. coli* accelerated even more advanced after 1997 when scientists published the entire genome of *E. coli*. The scientists surveyed all the 4288 genes present in it and discovered how their groups worked together to break down food, make new copies of DNA and performed other tasks.

In radiation biology, *E. coli* is widely used as a typical test system to screen the radioprotective activity (Diehl, 1995). This is normally done by various methods such as cell survival curve or dose response curve, survival curve of plasmid and also by study of genotoxicity assay in *E. coli*. Dose response curve is the easiest method to screen the radioprotective compounds. Dose response curve gives the radiation sensitivity of the organism with ionizing radiation which is carried out by plotting surviving fraction on Y-axis against radiation dose on X-axis. The main characteristics of such dose response curve is that; as the dose of radiation increased, a corresponding decrease in the probability of a cell is able to survive that dose or an increased in the reproductive death. The shape of this curve is characteristic for low LET (Linear Energy Transfer) radiation that is a kind of dose response that occurred in conventional radiotherapy. After an initial shoulder region, larger the dose on a linear scale, the smaller is the surviving fraction on a logarithmic scale (Diehl, 1995).

Inactivation Dose

When a population of microorganisms is irradiated with a low dose, only a few of the cells are damaged or killed. With increasing radiation dose the number of surviving organisms decreases exponentially (as it does with increasing heat treatment). Different species and different strains of the same species require different doses to reach the same degree of inactivation. In order to characterize organisms by their radiation sensitivity, the mean lethal dose (MLD) is normally used. It is the radiation dose required to kill 63% of a population, letting 37% to survive (D_{37}). A more commonly used measure of radiation sensitivity is the D_{10} dose, which is the dose, required to kill 90% of a population (Diehl, 1995).

If N_0 is the initial number of the organisms, N the number of organisms after the irradiation at a dose D and D_{10} the decimal reduction dose, then according to Diehl (1995) the equation can be defined as:

$$\text{Log}\frac{N}{N_0} = (-)\frac{1}{D_{10}} D$$

or

$$D_{10} = \frac{D}{\text{Log } N_0 - \text{log } N}$$

Dose response curve and D_{10} value can be found out by plotting dose of radiation on X-axis and percentage of survival on Y-axis.

The radioprotective ability of mussel hydrolysate on *E. coli* cells by plotting dose response curve and comparing with a known radioprotector as a positive control has been investigated in detail in the present study.

MATERIALS AND METHOD

Bacterial strain

E. coli ATCC 35218 was used for screening of the radioprotective activity of mussel hydrolysate.

Reagents and media

Brain Heart Infusion (BHI) broth, plate count agar and physiological saline were prepared according to the standard protocol.

Test Samples

Crude and aqueous mussel hydrolysate and cysteine were used as test samples. Required concentrations of all these test samples were prepared in terms of weight by volume ratio in phosphate buffer. Crude: 0.1 g/ml (10%), aqueous: 0.05 g/ml (5%) and cysteine: 0.005 g/ml (0.5%) were screened in the present study for the radioprotective activity.

Irradiation source

Gamma cell 220 (Atomic Energy of Canada Limited) of a dose rate of 9.0 Gy per minute was used for the present study.

Methodology

The bacterial culture was inoculated in BHI broth and incubated overnight at 100 rpm so as to get growth in the range of 10^8-10^9 cfu/ml. Ten fold dilutions of the culture were made in phosphate buffer to obtain 10^7 cells/ml. The cells were then incubated with mussel hydrolysate [crude (10%), aqueous (5%)], cystein (0.5%) for 30 minutes at room temperature of 25^0 C. Control was incubated with phosphate buffer solution. After incubation, 1.5 ml of the culture was added in eppendrof tubes and then irradiated at 100 Gy, 200 Gy, 300 Gy, 400 Gy and 500 Gy doses at melting ice temperature. Cells after irradiation were then plated on plate count agar after suitable serial dilutions and incubated at 37^0 C for 36 hours. The experiment was repeated for three times.

Radiation survival of *E . coli* (ATCC 35218)

The decimal reduction dose (D_{10}) of *E. coli* (ATCC 35218) was determined in aqueous and crude fractions of mussel hydrolysate. Cystein was also used as a positive control and the values were calculated by the following equation:

$$\text{Log}_{10} N/N_0 = -1/D_{10} \times D \qquad \text{(Diehl, 1995)}$$

Where D_{10} is the dose required to reduce population by a factor of 10, N= population after dose D, N_0 = initial population and D= dose in Gy. The $\log_{10} (N/N_0)$ plotted against dose D and showed a straight line relationship.

In another experiment, *E. coli* cells were incubated with all test samples and were irradiated with a dose of 300 Gy. The percentage of survival was calculated accordingly.

Methodology

E. coli was inoculated in BHI broth and incubated for over night on a shaker at 100 rpm. Logarithmically grown cells were harvested, washed and suspended in sterile 0.1 M phosphate buffer of pH 7.0 at the density of 10^6 cfu/ml. The cells were incubated with test samples for 30 minutes at room temperature. Control was incubated with phosphate buffer. After incubation, all samples were irradiated by a radiation dose of 300 Gy. The cells were then plated on plate count agar after suitable serial dilutions and surviving fractions were determined by scoring the colonies after 24 hrs of incubations at 35^0 C. The experiment was repeated for three times.

RESULTS

The radiation doses ranging from 0 to 300 Gy were initially used to select the appropriate dose of radiation to get D_{10} value with control cells (Table 2.1). It was found that an increase in dose of radiation by 100 Gy, the survival of the *E. coli* cells were reduced by 1 Log in each set of experiment. The D_{10} value in this experiment was 67.1211249 Gy. These values when plotted by taking radiation dose on X-axis and log of colony forming unit on Y-axis, a straight line was observed. The data were analysed statistically by applying regression equation and represented by y = -0.0149x + 7.4553; r^2=0.9995 (Fig. 2.1). Once the *E. coli* cells were treated with 10% crude mussel hydrolysate and doses ranging from 0 to 500 Gy were given, a significant increase in the value of D_{10} from 67.1211249 to 156.337 was observed (Table 2.2). A straight line relationship in all values in this experiment was also observed where y= -0064x + 7.2664; r^2=0.9928 (Fig. 2.2).

When *E. coli* cells were treated with 5% aqueous mussel hydrolysate and radiation doses ranging from 0 to 500 Gy were given, a significant increase in the value of D_{10} from 67.1211249 to 115.581 was observed (Table 2.3). A straight-line relationship in all values in this experiment was also observed in the present study (y= -0087x + 7.1839; r^2=0.9884) (Fig. 2.3).

Similarly when *E. coli* cells were treated with 0.5% cysteine and doses of radiation ranging from 0 to 500 Gy were given, a significant increase in the value of D_{10} from 67.1211249 to 211.021 was observed (Table 2.4). A straight-line relationship in all these values in this experiment was observed (y = -0047x + 7.4009; r^2=0.9269) (Fig. 2. 4).

It was found that when *E. coli* cells were incubated with test samples and irradiated with 300 Gy, both crude and aqueous fractions of mussel hydrolysate showed significant radioprotective activity. It was increased by 3 to 10% at designated concentrations of mussel hydrolysate. There was no further increase in radioprotective activity with increasing concentration of mussel hydrolysate Crude extract of 10% concentration showed nearly 27 times more protection than the control irradiated sample

whereas, aqueous extract of 10% concentration showed only 3 fold protection. However, cysteine of 0.5% concentration showed nearly 189 times more than the control irradiated cells (Table 2.5 and Fig. 2.6).

DISCUSSION

In recent years, *E. coli* has widely been used as a typical test system to screen the radioprotective activity of new compounds (Maniar *et al.*, 1984). For these studies, dose response curve using *E. coli.* has been reported to be the most convenient and easy method to screen the radioprotective ability of different compounds Several investigators have screened radioprotective compounds using *E. coli* as test system by studying the dose response curve or cell survival curve. Maniar *et al.* (1984) have screened the radioprotective activity of four phenothiazine drugs by studying the cell survival of *E. coli.* Fedorocko *et al.* (1994) reported that the pretreatment of mice with 50-1000 micrograms of the bacterial extract (Broncho-Vaxom) before sublethal irradiation found to induce an increase in the number of endogenous haemopoietic stem cells. Fluentes *et al.* (2006) showed that tannins from barks of *Pinus caribaea* protected the *E.coli* cells against DNA damage induced by γ-rays. Harada *et al.* (1999) have studied the sensitivity of Shuttle vector plasmid pZ189 DNA to carbon ion beams by using dose response curve. Imamura *et al.* (1999) have used cell survival curve to study the resistance of plasmid DNA to neon ion beams using transformation into recipient *E. coli* cells. Dergacheva *et al.* (1997) studied the combined effect of sodium nitrate and gamma rays on *E. coli* cells using cell survival curve. Korystov and Veksler (1986) reported the effect of oxygen and gamma rays on *E. coli* by means of cell survival curve.

There are many factors that effect the survival of microorganisms when irradiated with ionizing radiations. Deoxyribonucleic acids (DNA) in the chromosome represent the most critical target of ionizing radiation. Effect on the cytoplasmic membrane appears to play an additional role under some circumstances. Radiation sensitivity of macromolecules is roughly proportional to their molecular weight. Pollard (1975) has estimated that a dose of 0.1 kGy damaged about 2.8% of the DNA in bacterial cells, while the same dose damaged 0.14% of the enzymes and only 0.005% of the amino acids. The DNA damage of 2.8% is usually lethal to a large fraction of the irradiated cells and only few colonies are being found to develop upon inoculation of a culture medium.

Bacteria are less sensitive than mammals. The cells of the mammalian organisms containing DNA molecules provided much more genetic information than those of bacteria and more sensitive to radiation. Another factor influencing radiation effects is the structural arrangement of the DNA in the cell. During cell division, the double stranded DNA of bacteria separates and by means of a polymerase enzyme; new chains of DNA are assembled along the template. In the double stranded form, DNA is much less sensitive to radiation than in the single stranded form. D_{10} values can differ considerably in different media. Complex medium such as liquid egg provides a certain radiation protection to microorganisms as compared to irradiation in a buffer solution. D_{10} values even depend on whether the organisms are irradiated in low fat or in high fat medium. Possibly the radical scavenging properties of the proteins provide higher protection to the organisms in the high protein matrix. A dry medium such as desiccated coconut provides better protection than an aqueous medium such as liquid egg. D_{10} values in a frozen medium are usually higher than in a medium kept at room temperature during irradiation. The protective effect of drying and freezing can be explained as a result of the suppression of the indirect effects caused by the reactive intermediates (primarily $OH^{\cdot}$ radicals of water radiolysis).

The maximum protective and non-protective concentrations of chlorpromaizine and promethazine revealed that the number of single strand breaks in DNA was less at the protective concentration and it was efficiently repaired by the type-111 repair process. In the present study both crude mussels hydrolysate and aqueous hyrdolysate showed radioprotective activity on *E. coli* cells by increasing the D_{10} value when compared to the control irradiated cells. D_{10} value increased from 67 Gy of control to 156 Gy with crude mussel hydrolysate and up to 115 Gy with aqueous mussel hydrolysate. When *E. coli* cells were incubated with crude extract concentrations from 3 to 10% and irradiated with 300 Gy, there was significant concentration dependent radioprotecting activity.

In previous chapter, it has been mentioned that mussel hydrolysate contains important constituents such as albumen (22%), mineral salts (22%), microelements (iodine, copper, silver), macro elements (phosphorous, calcium ferrum), vitamins (B, B2, PP, AE), lipids melononidins and oligopeptides) (Boikov *et al.*, 1997). It has also been reported that the mussel hydrolysate possess immunomodulating property (Rao, *et al.*, 2002). Ability to protect *E. coli* by mussel hydrolysate from ionizing radiation might be related with complex mixture of various amino acids and metal ions present in the extract as studied by Rao *et al.* (2002).

Cystein is the earliest known radioprotector and a purified compound. It showed a high degree of radioprotection. Cysteine also acts as a free radical scavenger. Relatively mussel hydolysate showed lesser ability in reducing the effect of radiation when studied on *E. coli* cells. Interestingly the aqueous mussel hydrolysate showed less activity than the crude mussel hydrolysate. The aqueous mussel hydrolysate consisted of water content that it produced the free radicals on radiolysis and as such caused damage to bacterial cells. Thus our results confirmed that mussel hydrolysate posses a significant radioprotective ability. Further investigation will be required to find out the actual mechanism behind the radioprotective activity of mussel hydrolysate.

Table 2.1: *E. coli* cells irradiated with 100 Gy to 300 Gy (control)

Dose in Gy	Cfu	Log (Cfu/ml)	Slope	D10 (Gy)
0	30394900	7.482873584	-0.015	67.1211249
100	895000	5.951823035		
200	26300	4.419955748		
300	1065	3.027349608		

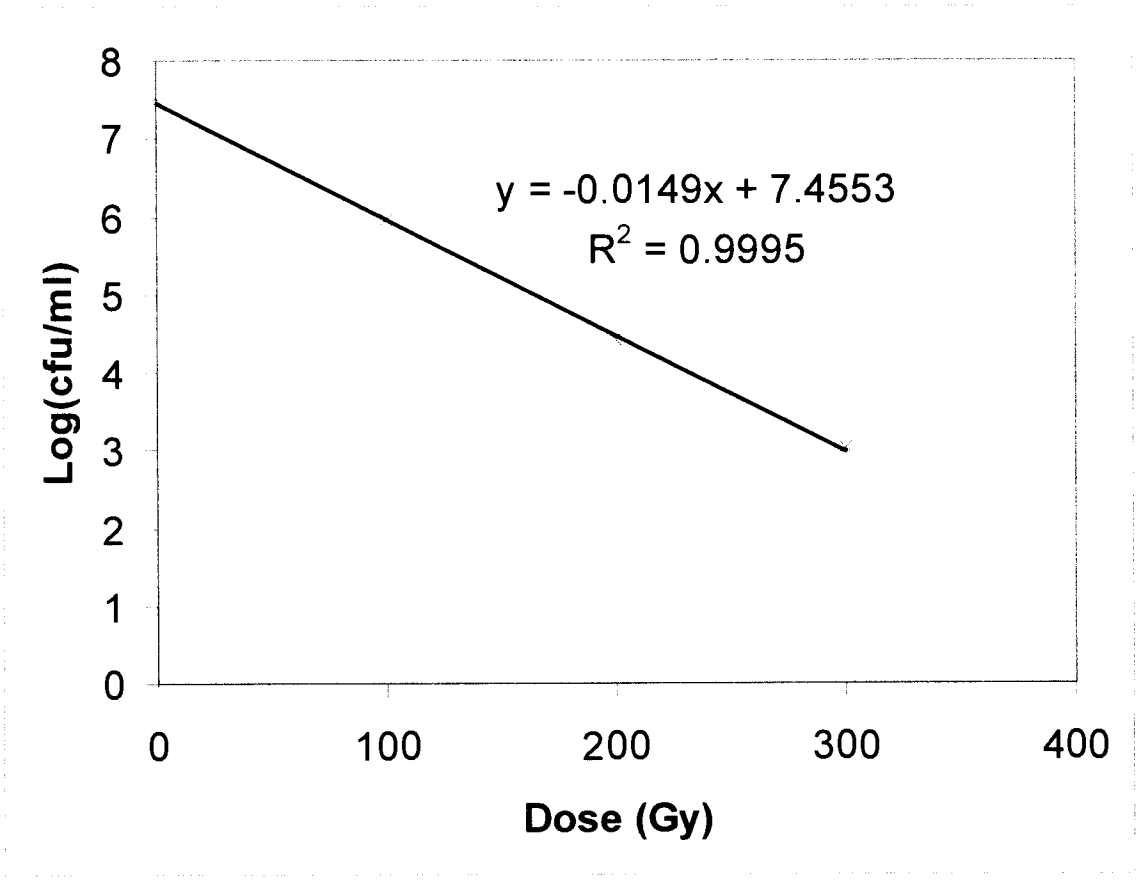


Fig. 2.1: Dose response curve of *E. coli* cells irradiated with 100 Gy to 300 Gy

Table 2.2: *E. coli* cells treated with 10% crude mussel hydrolysate and irradiated with 100 Gy to 500 Gy.

Cfu	log(Cfu/ml)	Slope	D10 (Gy)
23000000	7.36172784	-0.006	156.337
4500000	6.65321251		
645000	5.80955971		
218000	5.33845649		
47500	4.67669361		
14600	4.16435286		

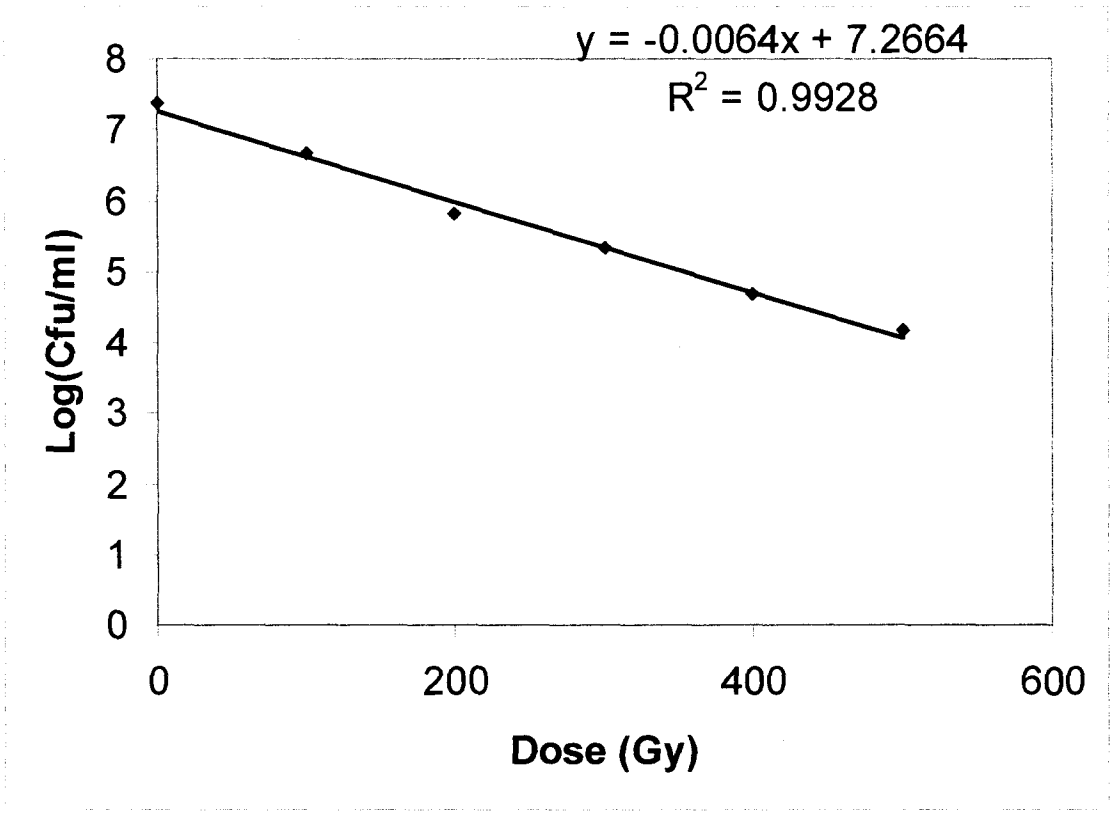


Fig 2.2: Dose response curve of *E. coli* cells treated with 10 % crude mussel hydrolysate and irradiated with 100 Gy to 500 Gy

Table 2.3: *E. coli* cells treated with 5% aqueous mussel hydrolysate and irradiated with 100 Gy to 500 Gy

Dose	Cfu	Log (Cfu/ml)	Slope	D10 (Gy)
0	25000000	7.39181692	-0.009	115.581
100	1800000	6.24797327		
200	207500	5.3170181		
300	22250	4.34733002		
400	7200	3.8573325		
500	920	2.96378783		

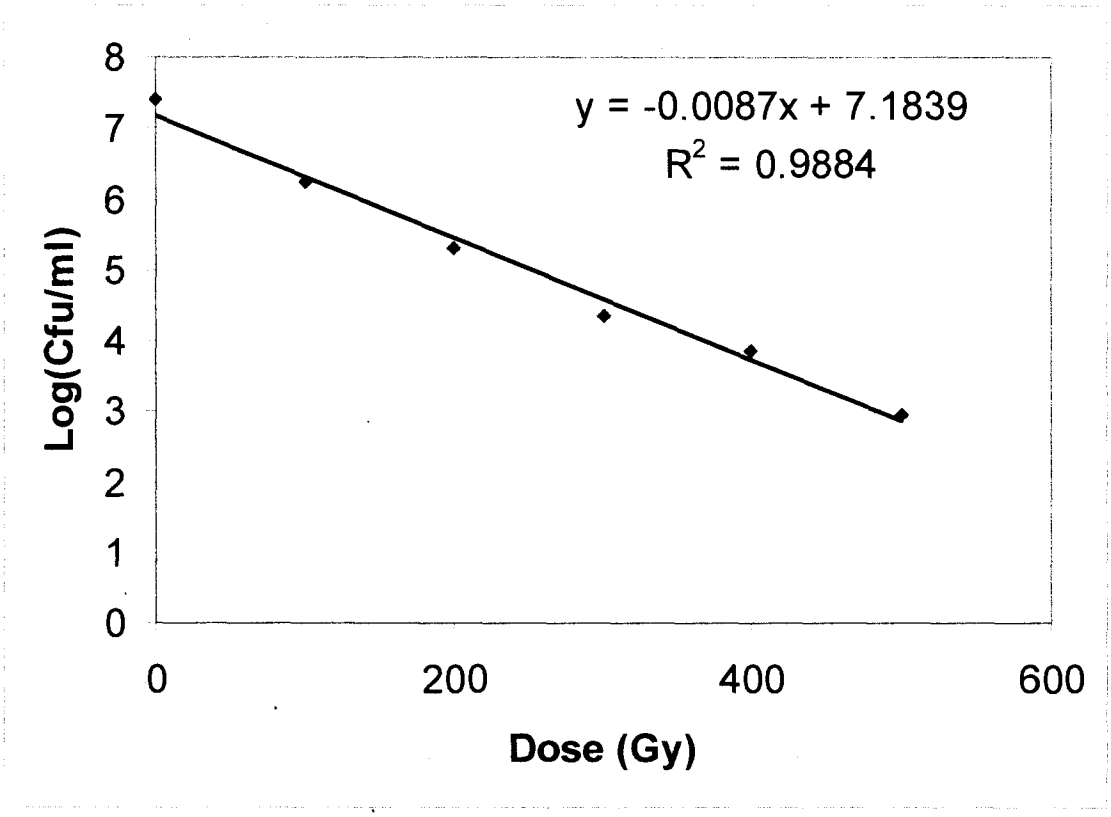


Fig. 2.3: Dose response curve of *E. coli* cells treated with 5% aqueous mussel hydrolysate and irradiated with 100 Gy to 500 Gy

Table 2.4: *E. coli* cells treated with 0.5% cysteine and irradiated with 100 Gy to 500 Gy

Dose	Cfu	Log (Cfu/ml)	Slope	D10 (Gy)
0	23000000	7.3569814	-0.005	211.021
100	7500000	6.87215627		
200	2500000	6.38291714		
300	1300000	6.10551018		
400	800000	5.90308999		
500	47500	4.67669361		

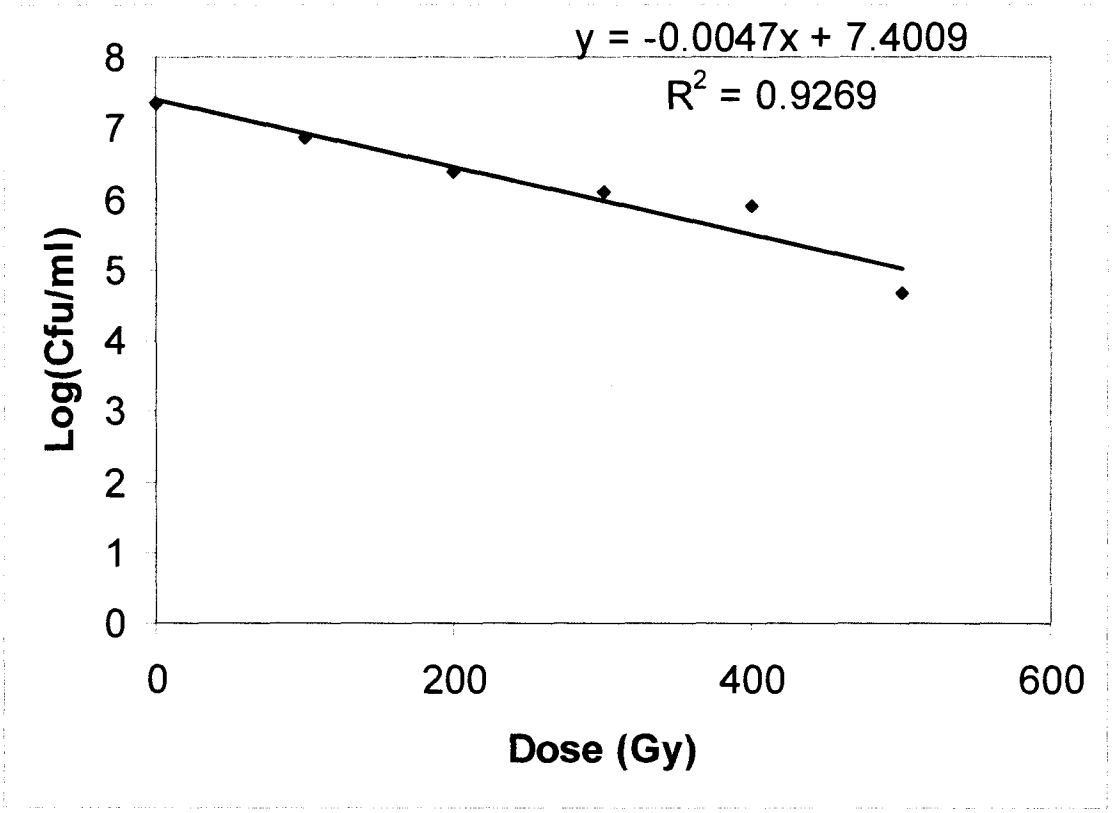


Fig. 2.4: Dose response curve of *E. coli* cells treated with 0.5% cysteine and irradiated with 100 Gy to 500 Gy

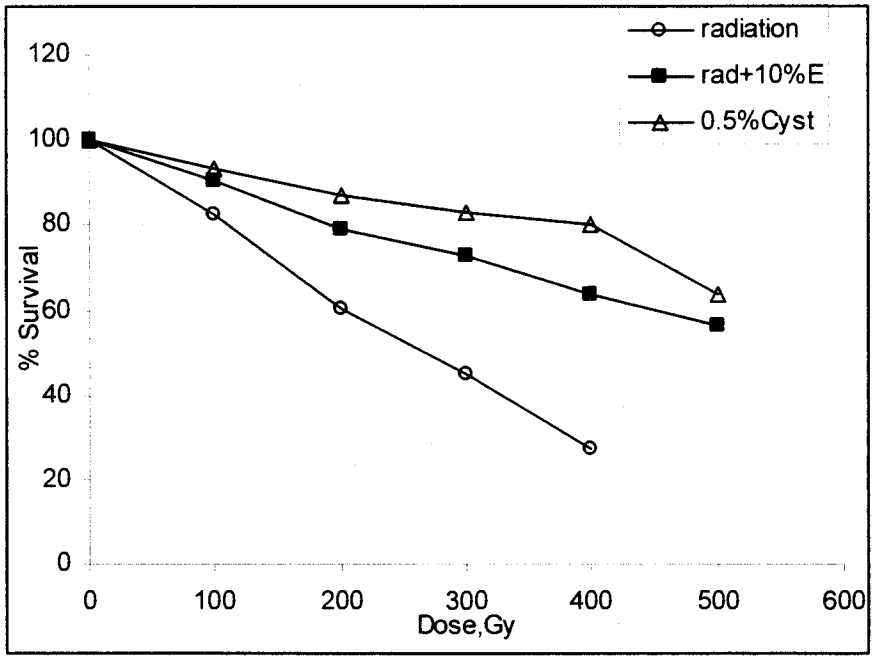


Fig. 2.5: Comparison of dose response curve of control irradiated, 10% mussel hydrolysate and 0.5% cysteine treated *E. coli* cells

Table 2.5: Radioprotective activity of mussel hydrolysate on *E. coli* irradiated with 300 Gy

Percentage of survival of *E. coli* in the test samples at a dose of 300 Gy							
Sample	**EXPT 1**	**EXPT 2**	**EXPT 3**	**EXPT 4**	**EXPT 5**	**Mean**	**S.D**
Control	100	100	100	100	100	100	
IRRADIATED	0.014	0.02	0.038	0.0096	0.0024	0.024	0.013
AQS (3%)	0.076	0.031	0.06	0.232	0.013	0.087	0.082
AQS (10%)	0.071						
CRUDE (3%)			0.90	0.51	0.22	0.543	0.341
CRUDE (5%)				0.544	0.318	0.431	0.159
CRUDE (10%)				0.736	0.57	0.65	0.117
CYSTEINE (0.5%)			8.37	3.1	2.2	4.55	3.33

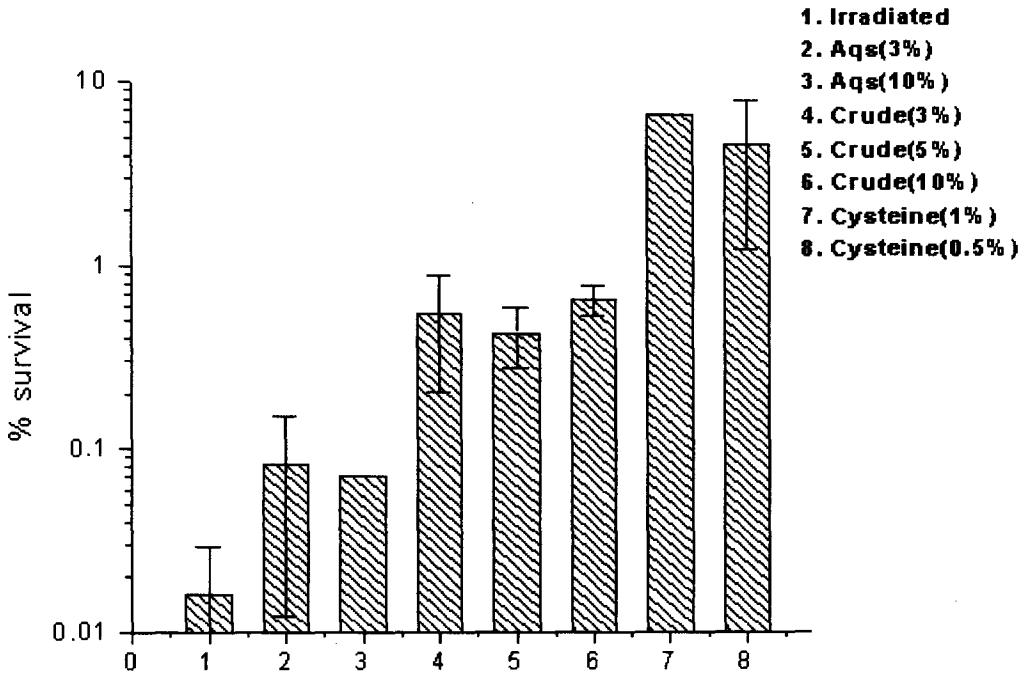


Fig. 2.6: Comparison of radioprotective activity of mussel hydrolysate with cysteine (% of survival calculated with respect to that of control)

CHAPTER 3

Radioprotective activity of mussel hydrolysate evaluated on yeast, *Saccharomyces cerevisiae*

Yeasts is considered as industrial microorganisms and used by man before the development of a written language. Hieroglyphics suggested that ancient Egyptians were using yeast in the process of fermentation to produce alcoholic beverages and leaven bread over 5,000 years ago. The biochemical process of fermentation responsible for these actions was not initially understood and undoubtedly looked upon by early man as a mysterious and even magical phenomenon. However, these early fermentation systems for alcohol production and bread making were formed by natural microbial contaminants of flour, other milled grains and from fruit or other juices containing sugar. Such microbial flora would have included wild yeasts and lactic acid bacteria that were found associated with cultivated grains and fruits. Over the course of time, the use of these starter cultures helped to select for improved yeasts by saving a "good" batch of wine, beer or dough for inoculating the next batch. For hundreds of years, it was traditional for bakers to obtain the yeast to leaven their bread as by-products of brewing and wine making. As a result, these early bakers have also contributed to the selection of these important industrial microorganisms.

Yeasts are single-celled fungi included edible mushrooms available at the supermarket. Common baker's yeast used to leaven bread, molds used to ripen blue cheese and production of antibiotics for medical and veterinary use. Many consider the edible yeast and fungi as natural as fruits and vegetables. Over 600 different species of yeast are known and they are widely distributed in nature. They are found in association with other microorganisms as part of the normal inhabitants of soil, vegetation, marine and other aqueous environments. Some yeast species are also natural inhabitants of man and animals. While some species are highly specialized and found only in certain habitats at certain times of the year, other species are generalists and can be isolated from many different sources.

Baker's yeast is used to leaven bread throughout the world and it is the type of yeast that people are most familiar as Baker's yeast. It is produced from the genus and species of yeast called *Saccharomyces cerevisiae*. The scientific name of the genus of baker's yeast, *Saccharomyces*, refers to "*saccharo*" meaning sugar and "myces" meaning fungus. The species name, *cerevisiae* is derived from the name Ceres - the

Roman goddess of agriculture. Baker's yeast products are made from strains of this yeast selected for their special qualities relating to the needs of the baking industry.

The typical yeast cell is approximately equal in size to a human red blood cell. These cells are spherical to ellipsoidal in shape. Because of its small size, it takes about seven billion yeast cells to make up to one gram of compressed Baker's yeast. Yeast reproduces vegetatively by budding, a process during which a new bud grows from the side of the existing cell wall. This bud eventually breaks away from the mother cell to form a separate daughter cell. Each yeast cell, on average, undergoes this budding process for 12 to 15 times before it is no longer capable of reproducing. During commercial production, yeast is grown under carefully controlled conditions on a sugar containing media typically composed of beet and cane molasses. Under ideal growth conditions, a yeast cell reproduces every two to three hours.

Yeasts can grow in the presence or absence of air. Anaerobic growth in the absence of oxygen is quite slow and inefficient. For instance, in bread dough, yeast grows very little. Instead, the sugar that can sustain either fermentation or growth is used mainly to produce alcohol and carbon dioxide. Only a small portion of the sugar is used for cell maintenance and growth. In contrast, under aerobic conditions, in the presence of a sufficient quantity of dissolved oxygen, yeast grow by using most of the available sugar for growth and producing only negligible quantities of alcohol. This means that the baker who is interested in the leavening action of carbon dioxide works under conditions that minimized the presence of dissolved oxygen. On the other hand, a yeast manufacturer who wants to produce more yeast cell mass, works under aerobic conditions by bubbling air through the solution in which the yeast is grown

In the production of baked goods, yeast is a key ingredient and serves three following primary functions:

A: Production of carbon dioxide

Carbon dioxide is generated by the yeast as a result of the breakdown of fermentable sugars in the dough. The evolution of carbon dioxide causes expansion of the dough as it is trapped within the protein matrix of the dough.

B: Causes dough maturation

This is accomplished by the chemical reaction of yeast that produced alcohols and physical stretching of the protein by carbon dioxide gas. These results in the light, airy physical structure associated with yeast leavened products.

C: Development of fermentation flavor

Yeast imparts the characteristic flavor of bread and other yeast leavened products. During dough fermentation, yeast produces many secondary metabolites such as ketones, higher alcohols, organic acids, aldehydes and esters. Some of these, alcohols for example, escape during baking. Several react with each other and with other compounds found in the dough, which form new and more complex flavor compounds. These reactions occur primarily in the crust and the resultant flavor diffuses into the crumb of the baked bread.

The basic carbon and energy source for yeast growth are sugars. Starch cannot be used because yeast does not contain the appropriate enzymes to hydrolyze this substrate to fermentable sugars. Beet and cane molasses are commonly used as raw material because the sugars present in molasses, a mixture of sucrose, fructose and glucose, are readily fermentable. In addition to sugar, yeast also requires certain minerals, vitamins and salts for growth. Some of these can be added to blend the beet and cane molasses prior to flash sterilization while others are fed separately to the fermentation. Alternatively, a separate nutrient feed tank can be used to mix and deliver some of the necessary vitamins and minerals. Required nitrogen supplied in the form of ammonia and phosphate is supplied in the form of phosphoric acid. Each of these nutrients is fed separately during fermentation to permit better pH control of the process. The sterilized molasses, commonly referred for as mash or wart, is stored in a separate stainless steel tank. The mash stored in this tank is then used to feed sugar and other nutrients to the appropriate fermentation vessels

The process by which baker's yeast is obtained in bulk quantities, has been the major factor in ensuring the popularity of *Saccharomyces cerevisiae* as an experimental organism in biochemical studies. This yeast species has another advantage of having a sexual cycle and so is amendable to genetical analysis. For the past two decades *S. cerevisae* has been the model system for much of molecular genetic research because the basic cellular mechanics of replication, recombination, cell division and metabolism are generally conserved between yeast and larger eukaryotes, including mammals. In radiation biology, yeast has been used as a typical test system to study the radioprotective effect of various compounds by plotting cell survival curves. Cell survival curves are used to show the response of single cells to increasing single doses of radiation of various qualities delivered under various environmental conditions and at various rates of dose. The response of single cell is tested by their ability to grow into colonies (i.e. to proliferate) after that treatment. If a cell demonstrates this reproductive capacity during a minimum period after the treatment, then the cell can be considered to have survived. In this context, survival requires not merely the continued existence of the cell as a living entity (functionally intact) but also the property of proliferation.

Dose response curves *in vitro*

Compared to the number of colonies in an un-irradiated aliquot of cells (defined as 100% or unity), the number of colonies formed in an irradiated aliquot is represented the fraction of the initial cell number that survived after irradiation. The ability of single cell to proliferate into colonies was used by Puck and Marcus (1956) in a plating technique analogous to that used for bacterial cell viability. Dose response curve was made by plotting surviving fraction on Y-axis against radiation dose on X-axis. Characteristics of such dose response curve can be defined as "the dose of radiation increases, a corresponding decrease in the probability of a cell to survive that dose, i.e. an increase in the reproductive death". The shape of this curve is characteristic for low LET (Linear Energy Transfer) radiation, i.e. it is the sort of dose response that is found with conventional radiotherapy. After an initial shoulder region, larger the dose on a linear scale, the smaller the surviving fraction on a logarithmic scale.

Many authors have used dose response curve to study the radioprotective activity of different compounds in yeast - *Saccharomyces cerevisiae*. Pasupathy (1998) used yeast respiratory adaptation system to detect chemical mutagens / carcinogens in mammals. Pasupathy *et al.*, (2001) have studied the effect of AK2123, (Sanazole) a hypoxic radio-sensitizer on yeast *Saccharomyces cerevisiae*. Vaidya and Pasupathy (2001) have used *Saccharomyces cerevisiae* as a test system to study radioprotective action of caffeine. A number of antioxidants were tested for their efficacy in radioprotection using yeast cells (Vaidya *et al.*, 2004). Bala and Goel (2004) have studied the radioprotective effect of podophyllotoxin in *Saccharomyces cerevisiae*. Petin and Matrenina (1981) studied the radioprotective action of cysteine and cysteamine on gamma-irradiated yeast cells of various genotypes. In the present study, *Saccharomyces cerevisiae* was used as a test system to study the radioprotective effect of mussel hydrolysate by plotting dose response curve. Radioprotective activity of mussel hydrolysate was also studied in a concentration dependent manner.

MATERIALS AND METHODS

Reagents

The chemicals such as sodium dihydrogen phosphate, disodium hydrogen phosphate, bacto-yeast extract, bactopeptone, dextrose, bacto-agar were obtained from Himedia for the present study.

Preparation of the media

Yeast extract peptone dextrose media was prepared according to following standard protocol:

Composition	Percentage
Yeast extract	1
Peptone	2
Dextrose	2
Bacto agar	2

Preparation of the test sample

Mussel hydrolysate of 0.05 g/ml was prepared in terms of weight by volume ratio in phosphate buffer solution.

Strains and Growth conditions

Haploid strains of the yeast *Saccharomyces cerevisiae,* Wild type ATCC 3177 used for the present study were the kind gifts of Prof. R. Haynes, Department of Biology, and York University, Ontario, Canada. Cells were cultured in yeast extract-bacto peptone-dextrose (YEPD, broth) at 30^{O} C with constant shaking. Overnight grown cells in log phase were used for the study.

Irradiation source

Gamma cell 220 (Atomic Energy of Canada Limited), with a dose rate of 5.9 Gy per minute was used for the present study.

Methodology

Yeast cells were inoculated in YEPD broth and incubated for over night at 30^0 C on a rotary shaker at 100 rpm. Logarithmically grown cells were harvested, washed and suspended in sterile 0.1 M sodium phosphate buffer (pH 7.4) at the density of about 10^6 cells/ml. Cells were then incubated with mussel hydrolysate at a concentration of 0.05 g/ml for 30 minutes at 4^0 C. Control was incubated with phosphate buffer. After incubation, all samples were irradiated with γ-radiation dose of 150 Gy to 1050 Gy in a Gamma Cell 220 with a dose rate of 5.9 Gy/min at ice-cold condition.

In another set of experiments, yeast cells were incubated with 0.01 g/ml (1%), 0.02 g/ml (2%), 0.03 g/ml (3%), and 0.05 g/ml (5%) for 30 minutes and then they were irradiated with a dose of 250 Gy.

Determination of cell survival

Cells after irradiation were plated on YEPD agar after suitable serial dilutions with phosphate buffer and the surviving fractions were determined by scoring macroscopic colonies after three days of incubation at 30^0 C. The experiment was repeated three times.

Survival curve was made by plotting different doses of radiation on X-axis and percent survival on Y-axis. From the survival curve, D37 value, the dose required to reduce the survival of the colony from 100 to 37% were obtained for both control irradiated and the mussel treated samples (Test samples). Similarly, the DMF (Dose Modifying Factor) values (the ratio of D37 of control irradiated one to the test samples) were calculated for evaluating the indicative of radioprotective activity of test sample. DMF value was calculated using the following equation:

$$\text{Dose Modifying Fraction (DMF)} = \frac{\text{D37 value of control irradiated}}{\text{D37 value of test sample}}$$

RESULTS

The percentage of survival of yeast cells was decreased from 65.98 to 3.50% when the cells were irradiated with radiation dose from 150 Gy to 1050 Gy. There was a clear dose dependent pattern in the survival of cells with concentration of radiation dose from 150 to 1050 Gy (Table 3.1). The D37 value of 355 was obtained for the control-irradiated sample. On the contrary, when the yeast cells were incubated with mussel hydrolysate (concentration: 0.05 g/ml) and irradiated with radiation dose from 150 to 1050 Gy, the D_{37} value was 415 (Table 3.2) showing significant survival of yeast cells.

A comparison of percentage of survival of yeast cells in control irradiated and cells treated with mussel hydrolysate was done to show the radioprotecting activity. A significant increase in the survival of cells treated with mussel hydrolysate was observed (Table 3.3). When yeast cells were incubated with 1 g/ml to 5 g/ml of mussel hydrolysate and irradiated with 250 Gy, there was a significant protection of cells from 31.5 to 48.46% (Table 3.4). Maximum radioprotection activity was found with mussel hydrolysate at a concentration of 0.05 g/ml.

Comparison of dose response curves in control irradiated and treated group with mussel hydrolysate were presented in Figure 3.1. The Dose Modifying Factor of 0.855 for mussel hydrolysate was calculated as follows:

$$\text{Dose modifying factor} = \frac{355}{415} = 0.855$$

where 355 is the D_{37} value for control and 415 D_{37} value for test sample.

DISCUSSION

Yeasts are simple unicellular fungi and referred more to a life-style than to a phylogenetic classification. The most well-known and commercially known yeast is *Saccharomyces cerevisiae,* often called as common baking or brewing yeasts which are actually the strains of *Saccharomyces cerevisiae (*Fowell *et al.,* 1969). Hydrolysate extract from *Saccharomyces cerevisiae* helps to prevent bacterial and fungal diseases in treated plants. It appears to act by enhancing the plant's natural defense mechanisms. The active ingredient is approved for use on all food crops as well as on turf and ornamental plants. Yeast extract is a common food flavoring and possess a long history as a plant fertilizer. They are not harmful to human health or the environment. The active ingredient consists primarily of oxidized amino acids, but it also includes nutrients such as vitamins and minerals. The active ingredient from yeast is cleared by the U.S. Food and Drug Administration (FDA) as a flavor enhancer for soups, fruits and other foods. Because yeast extract is especially rich in B vitamins, it has also been used as a human nutritional supplement. Yeast extract from *Saccharomyces cerevisiae* has been reported to stimulate the natural defense mechanisms in plants (Fowell *et al.,* 1969). The end product also improves growth, yield and shelf life of crops.

The problem posed to the yeast manufacturer, however, is not as simple as just adding air during the fermentation process. If the concentration of sugar in the fermentation growth media is greater than a very small amount, the yeast will produce some alcohol even if the supply of oxygen is adequate or even in abundance. This problem can be solved by adding the sugar solution slowly to the yeast throughout the fermentation process. The rate of addition of the sugar solution must be such that the yeast uses the sugar quickly. This type of fermentation is referred to as fed-batch fermentation.

In radiation biology, yeast has been used as a typical test system to study the radioprotective effect of various compounds by plotting cell survival curves. The yeast model is sensitive, rapid, simple and convenient to show the distribution of chemical mutagens or carcinogens (Pasupathy, 1999). This method has also effectively been used by several workers to study the DNA acting potentiality of variety of chemicals (Nagalakshmi *et al.*, 1984; Pasupathy and Pradhan, 1999). The mechanism of action of some of the radioprotective compounds has been well documented by several authors (Murthy and Pasupathy, 1995). The replication of duplex DNA is highly complex phenomenon where coordination of the process of replication involved action of several enzymes in unison (Kornberg and Baker, 1992). Detectable concentrations of chemical and their DNA-acting metabolites have been reported to be analysed easily in urine, liver, lungs, kidney and spleen (Pasupathy, 1999).

In the process of detecting efficacy of a compound using yeast is well elucidated where the test compound initially incubated with the yeast cells for 15 minutes followed by proper washing. This process has been observed to distinguish a DNA acting compound from inhibitors of cellular processes like RNA, protein synthesis or respiration which are needed to be present continuously to be effective (Pasupathy and Pradhan, 1999).

A number of rapid screening models using micro-organisms have been reported by Zimmerman (1973). Pasupathy (1999) further demonstrated the use of yeast for the rapid detection of chemical mutagens or carcinogens to show the distribution of an ingested mutagen / carcinogen on mammalian model. A screening system basically on respiratory adaptation (*de novo* biogenesis of mitochondria) during transition from anaerobic to aerobic mode of growth in *S. cerevisiae* has been reported (Zimmerman, 1973). Nonetheless, respiratory adaptation in *S. cerevisiae* is highly susceptible to DNA – acting compounds on account of damage to mitochondrial DNA in anaerobic cells. These are the methods where both parent compounds as well as their metabolites formed by the action of yeast are effectively be detected (Pasupathy, 1999).

The present study showed that yeast cells were protected against γ-radiation-induced killing by mussel hydrolysate significantly. Mussel hydrolysate increased the DMF value from 355 to 415 Gy giving a DMF value of 0.855, which is indicative of its radioprotective activity. When yeast cells were incubated with mussel hydrolysate of 0.01 g/ml to 0.05 g/ml and radiated with a dose of 250 Gy, a maximum protection with 0.05 g/ml of mussel hydrolysate was found to be 48%. There has not been any toxicity observed in mussel hydrolysate. Vaidya and Pasupathy (2001) have screened the radioprotective activity of caffeine in *S. cerevisiae* and found that caffeine protected the yeast cells under aerobic condition and have a DMF value of 0.33. They also found that caffeine protected yeast cells by reducing the radiation damage rather than by interfering with DNA repairing process. These observations support the potential in mussel hydrolysate for protecting the microorganisms from the radiation damages.

Table 3.1: Yeast cells irradiated with dose of 150 Gy to1050 Gy (Control)

Dose in Gy	Percent survival				
	Expt 1	Expt 2	Expt 3	Expt 4	Mean
0	100.00	100.00	100.00	100.00	100.00
150	65.45	77.30	61.17	60.00	65.98
300	56.36	49.49	42.82	49.33	49.50
450	20.36	28.69	31.12	35.11	28.82
600	18.18	8.60	17.29	14.69	14.69
750	15.63	6.10	12.77	11.50	11.50
900	14.90	6.00	9.90	11.00	10.45
1050	4.00	3.50	3.50	3.00	3.50

Table 3.2: Yeast cells incubated with mussel hydrolyzate concentration of 0.05 g/ml and radiated with dose of 150 Gy to 1050 Gy

Dose in Gy	Percent survival			
	Expt 1	Expt 2	Expt 3	Mean
150	81.78	80.00	74.54	78.77
300	63.56	66.60	63.63	64.60
450	34.67	35.50	30.90	33.69
600	20.22	26.60	25.45	24.09
750	6.67	17.55	20.00	14.74
900	6.00	16.00	74.54	11.00
1050	5.96	14.60	10.28	10.28

Table 3.3: Comparison of survival of yeast cell incubated with mussel hydrolysate and control, irradiated at dose of 150 Gy to 1050 Gy

Dose in Gy	Percent survival	
	Mussel hydrolyzate (0.05 g/ml)	Control
0	100.00	100.00
150	78.77	65.98
300	64.60	49.50
450	33.69	28.82
600	24.09	14.69
750	14.74	11.50
900	11.00	10.45
1050	10.28	3.50

Table 3.4. Radioprotective activity of mussel hydrolysate on yeast cells irradiated with 250 Gy.

Sample (g/ml)	Colony Number		Survival (%)	Protection (%)
	Before radiation	After radiation		
Control	245	120	49.27	-
0.01	245	176	72	31.5
0.02	240	206	86	42.7
0.03	245	230	94	47.0
0.05	245	232	95.6	48.46

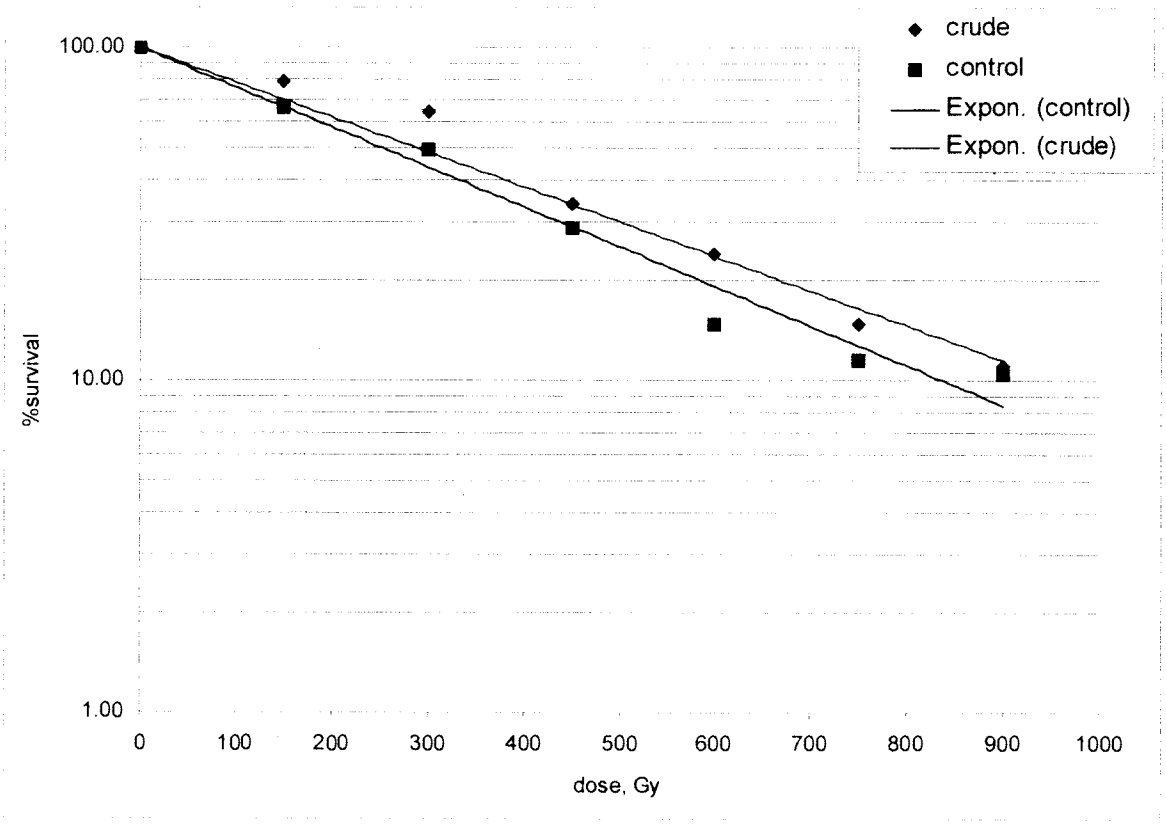


Fig 3.1: Radioprotection of yeast cells by crude mussel hydrolysate

CHAPTER 4

Evaluation of free radical scavenging activity of mussel hydrolysate on mice lymphocytes

Atoms are most stable in ground state and they are considered to be "ground" when every electron in the outermost shell has a complimentary electron that spinned in the opposite direction (Karlsson, 1997). A free radical is an atom like oxygen and nitrogen consisting of at least one unpaired electron in the outermost shell showed an independent existence (Karlsson, 1997). Free radicals are chemical species possessing an unpaired electron in the outer (valence) shell of the molecule or an atom or group of atoms that has at least one unpaired electron as such they are unstable and highly reactive (Halliwell, 1996). This species is in reality composed of a group of molecular fragments that are capable of independent existence (Cheeseman and Slater, 1993).

A free radical is easily formed when a covalent bond between entities is broken and one electron remains with each newly formed atom (Karlsson, 1997). Free radicals are highly reactive due to the presence of unpaired electron(s). When free radicals steal an electron from a surrounding compound or molecule, a new free radical is formed in its place. In turn, the newly formed radical then looks to return to its ground state by stealing electrons with antiparallel spins from cellular structures or molecules. Thus the chain reaction continues (Goldfarb, 1999). Any free radical involving oxygen can be referred to as Reactive Oxygen Species (ROS). Reactive Oxygen Species include a number of chemically reactive molecules derived from oxygen (Halliwell, 1996; Fridovich, 1999; Halliwell, 1999; Betteridge, 2000). Oxygen centered free radicals contain two unpaired electrons in the outer shell. The seemingly paradoxical consequences of the beneficial and harmful effects of oxygen (O_2) have been shown for several decades (Halliwell and Gutteridge, 1984). Free radicals can produce from both exogenous and endogenous sources. They are produced continuously in cells either as by-products of metabolism or deliberately as in phagocytosis (Cheeseman and Slater, 1993).

The mitochondria are a major site of generation of reactive oxygen species where electrons leak out to generate superoxide radical (Boveris and Chance, 1973). In aerobic organisms, oxygen (O_2) is reduced to water (H_2O) at the end of the mitochondrial respiratory chain. The O_2 molecule remains bound to ferrocytochrome c:oxygen oxidoreductase (complex IV of the mtiochondrial respiratory chain) until it is fully reduced to H_2O by the transfer of four electrons and two protons. However, further

upstream in the mitochondrial respiratory chain (complex 1, 11 and/or 111), there is leakage of single electrons mainly from the nonheme iron-sulphur proteins, leading to the partial reduction of O_2 to form the superoxide anion ($O_2^{\cdot -}$). Super oxide is a radical and a chemical species with an unpaired electron (Kehrer and Smith, 1994). Radicals tend to be reactive species because electrons like to pair up to form stable two-electron bonds. Therefore, $O2^{\cdot -}$ is called a Reactive Oxygen Species (ROS). Superoxide can further be reduced by the addition of a second electron to hydrogen peroxide (H_2O_2), which is not a radical. The addition of a third electron leads to the formation of the hydroxyl radical ($^{\cdot}OH$), which is the most reactive and aggressive of the various ROS. All these reactive oxygen species are otherwise known as Oxygen Free Radicals (OFR).

In addition to $O_2^{\cdot -}$, H_2O_2, and $^{\cdot}OH$, there is another type of ROS called singlet oxygen (O_2) (Frei, 1994). Singlet oxygen can be formed from ground state triplet oxygen in presence of a photosensitizer and light. It has been reported that during the normal aerobic metabolism, the leakage of electrons from the mitochondrial respiratory chain to yield $O_2^{\cdot -}$ occurs continuously. It has also been estimated that 2-5% of all the electrons that travel down the respiratory chain, never made it to complex IV, but instead leaked from the respiratory chain to formed $O_2^{\cdot -}$ and its dismutation product H_2O_2 (Boveris and Chance, 1973). In addition to the mitochondrial and other cellular electron transport systems, there are further endogenous sources of $O_2^{\cdot -}$ production which included reactions of lipoxygenases, cyclo-oxygenases, soluble oxidase enzymes such as reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase in phagocytic cells (neutrophils and monocytes), D-amino acid oxidase and xanthine oxidase (Cross and Jones, 1991); epinephrine and quinoid substrates, such as coenzyme Q_{10} and vitamin K which normally undergo redox cycling and the cytochrome P450 system (Ames *et al.*, 1993; Keher and Smith, 1994).

Once free radical forms from the various endogenous sources, $^{-}O_2^{\cdot}$ can react further and dismutate to H_2O_2 and O_2 in the presence of catalytic amounts of iron and copper and further form $^{\cdot}OH$ in the "metal catalysed Haber-Weiss reaction" (Frei, 1994). This reaction is the sum of two separate reactions; the first being the reduction of ferric or cupric ions (Fe^{3+} or Cu^{2+}) by $O_2^{\cdot -}$, and the second is the reaction of the reduced metal ions (ferrous or cuprous, Fe^{2+} or Cu^{+}) with H_2O_2 to form $^{\cdot}OH$ radical through Fenton reaction (Frei, 1994). The sequence of reactions shown in the Figure 5.1 showed

that $O_2^{\cdot -}$ that helped in forming other ROS, including ·OH, provided the availability of a redox- active metal catalyst (Fig. 4.1).

$$2\ O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$
$$O_2^{\cdot -} + H_2O_2 + H^+ \text{ metal catalyst } O_2 + {\cdot}OH + H_2O$$
$$[\ (O_2^{\cdot -} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$
$$H_2O_2 + H^+ + Fe^{2+} \rightarrow {\cdot}OH + H_2O + Fe^{3+})]$$

(Frei, 1994)

Fig. 4.1

The oxidation potential and reactivity of various ROS may be given in the following order (Fridovich, 1978):

$$O_2^{\cdot} < H_2O_2 < O_2 < {\cdot}OH$$

Sources of Reactive Oxygen Species

Reactive oxygen species are produced by both endogenously and exogenously (Fridovich, 1978). All aerobic cells generate reactive oxygen species endogenously by products of a number of metabolic reactions (Fridovich, 1978). Endogenous sources include Electron Transport Chain (ETC), activated leukocytes, enzymes, quinoid substrates (drugs, diet, pesticides) and exogenous sources include indoor and outdoor air (cigarette smoke, radon, O_3, NO_2, SO_2, car exhaust etc), ionizing radiations like X-rays, gamma rays, UV light, and environmental pollutants (Trush *et al.*, 1982; Riley, 1994).

Reactive oxygen species have both beneficial and adverse effects. Vital beneficial physiological cellular use of ROS is now being demonstrated in different areas including intracellular signaling and redox regulation. Hydrogen peroxide and superoxide act as regulator of transcription factor activities and other determinants of gene expression (Sundaresan *et al.*, 1995; Finkel, 1998; Kamata and Hirata, 1999; Rhee, 1999; Patel *et al.*, 2000). Several cytokines, growth factors, hormones and neurotransmitters use ROS as secondary messengers in the intracellular signal

transduction (Thannickal and Fanburg, 2000). When phagocytes are activated, they produce ROS in enough amounts to kill intruding bacteria (Thomas *et al.,* 1988). These ROS include superoxide, hydroxyl radical and hypochlorous acid. The two highly reactive ROS molecules thereby formed in phagosomes (HOCL and $OH^{\cdot}$) are highly toxic to bacteria ingested by the phagocyte and carry the direct antimicrobial effects of ROS.

However, excess generation of ROS in cells is known to damage DNA, lipids and proteins resulting in several biological effects ranging from alterations in signal transduction and gene expressions to mitogenesis causing to cell death (apoptosis) (Keher, 1993; Wiseman and Halliwell, 1996; Berlett and Stadtman, 1997; Hunt *et al.,* 1998; Rollet *et al.,* 1998; Kannan and Jain, 2000). Reactive oxygen species are potentially toxic, mutagenic or carcinogenic. To counter this, cells have acquired endogenous antioxidant defence system, which scavenge ROS at different levels (Heng *et al.,* 2000) either directly by interception or indirectly through the reversal of oxidative damage. This includes antioxidant enzymes like Superoxide Dismutase (SOD), Catalase, Glutathione Peroxidase (GPX) and low molecular weight antioxidant substrates like Vitamin E and C, different selenium compounds, β carotene, lipoic acid, ubiquinones etc. When ROS overcome the defence systems of the cell and redox homeostasis is altered, it resulted in oxidative stress. It is reported that the generation of reactive oxygen species or reactive nitrogen species can cause cell death either by apoptosis or necrosis (Kannan and Jain, 2000). Oxidative stress is implicated in the pathogenesis of several disease including AIDS, Huntington's, Parkinson's disease, amyotropic lateral sclerosis, Alzheimer's disease and retinal degenerative disorders (James *et al.,* 2002).

Tempol a low molecular weight water-soluble nitroxide shown to permeate the cell membrane freely and act as a superoxide dismutase mimic (Hahn *et al.*, 1999). Scavenging of free radicals, induction of hypotension and bone marrow hypoxia are thought to be reason for radioprotection of Tempol (Hahn, *et al.,* 1998; 1999). Captopril (D-3-mercapto-2-methylpropanoyl-L-proline) which is an Angiotensin Converting Enzyme (ACE) showed radioprotective activity due to its free-radical scavenging activity and protease inhibition (Molteni *et al.*, 2000). Several medicinal plant products have also been effective as free radical scavengers (Maurya *et al.,* 2006). Caffeine has been found

to scavenge hydroxyl radical and competes with oxygen for radiation-induced electrons (Singh and Kesavan, 1990; Devasagayam *et al.*, 1996; Kesavan, 2005). Ferulic acid is a monophenolic phenylpropanoid compound that occurs in plant products such as rice, green tea and coffee beans. These compounds have been found to scavenge the reactive oxygen species such as hydroxyl radical (OH), hypochlorous acid (HOCl) and peroxyl radical (RO_2) (Scott *et al.*, 1993) and stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Kikuzaki *et al.*, 2003). Glycyrrhizic acid (GZA), which is a root extract of the plant *Glycyrrhizia glabra,* offered radioprotection by scavenging free radicals (Gandhi *et al.*, 2004). Troxerutin, a derivative of the natural flavonoid has been found to scavenge oxygen-derived free radicals (Wenisch, 2001; Kessler *et al.*, 2002).

Measurement of free radicals

There are many ways of studying free radicals but these methods have to deal with the major problems associated with their high reactivity mainly their relative short half-lives and migration distances. These features make the measurement of free radicals very difficult. Oxygen Free Radicals (OFR) can only be studied directly *in vitro* by physio-chemical methods such as Electron Spin Resonance (ESR). For practical reasons, such methods are limited to *in vitro* studies. OFR can be measured *in vivo* by 'trapping' them using spin traps which can be measured for in *ex vivo* studies by ESR. Similarly they can be trapped *in vivo* and detected *ex vivo* by their reaction with other chemicals such as salicylic acid. Utilizing the fact that reaction involving OFRs, gives rise to chemiluminescence and allows their reaction to be monitored both *in vitro* and *in vivo* in exposed organs.

Electron spin (or paramagnetic) resonance (ESR or EPR) is a spectroscopic technique that detects the unpaired electron present in a free radical. It is the only general approach that can provide direct evidence for the presence of a free radical. In addition, analysis of the ESR spectrum generally enables determination of the identity of the free radical.

Although the ESR is highly sensitive, it is not directly applicable to the study of biological oxidations or to the majority of radical chemistry. A more successful technique, commonly known as spin trapping' method which permit ESR investigation of short lived reactive free radicals by transforming them into more persistent species. This method is mainly used for studying the hydroxyl radical.

2'-7 Dichlorodihydrofluorescein diacetate (DCFH-DA) is the other widely used techniques for directly measuring the redox state of a cell (James *et al.,* 2002). This is a cell permeable relatively nonfluorescent molecule and posses many advantages over other techniques, so far, developed. This technique is easy to use, extremely sensitive to changes in the redox state of a cell, inexpensive and used to follow changes in ROS over time. Activity of cellular esterases cleaves with DCFH-DA into 2'-7 Dichlorodihydrofluorescein ($DCFH_2$). The basic principal involves in this method is based on the fact that Peroxidases, cytochrome c and Fe^{2+} can all oxidize $DCFH_2$ to 2'-7 Dichlorofluorescein (DCF) in presence of hydrogen peroxide. Accumulation of DCF in cells is being measured by an increase in fluorescence at 530 nm when the sample is excited at 485 nm. Fluorescence at 530 nm is measured using a flow cytometer and it is assumed to be proportional to the concentration of hydrogen peroxide in the cells (Bass *et al.,* 1983; Royall and Ischiropoulos, 1993). Thus fluorescence intensity serves as an indicator of ROS generation. The fluorescence intensity increases with the increase in the ROS generation.

The technique of pulse radiolysis was introduced in 1960 by McCarthy and McLaughlan (1960) and ten years later after the invention of the light equivalent flash photolysis by Porter (1950). Some of the earlier uses of pulse radiolysis in the biologically relevant free radical field involved studies on the superoxide radical. Pulse radiolysis was able to show conclusively that superoxide dismutase (SOD) did in fact efficiently dismutate the radicals. The method relied on the ability of pulse radiolysis to rapidly produce a known concentration of superoxide radicals in a very short period of time and can be used for many different studies on these radicals.

Free radical scavenging activity can be studied indirectly by means of spectroscopic methods. There have been many methods for measuring free radical production in cells such as chemilusminescence of luminol (Suzuki *et al.,* 1998; Dahlgren and Karlsson, 1999; Mellqvist *et al.,* 2000) and lucigenin (Gyllenhammar, 1987), cytochrome c reduction (Dahlgren and Karlsson, 1999), ferrous oxidation of xylenol orange (Nourooz-Zadeh, 1999) and DCFH-DA (Carmody and Cotter, 2000; Tammariello *et al.,* 2000; Ottonello *et al.,* 2001). These are the most straightforward technique used by cell permeable fluorescent and chemilusminescent probes. Flow cytometry or fluorimetry are the other technique used for the detection of ROS with fluorescent probes.

Several authors have used fluorescence method to detect the reactive oxygen species levels in cells by means of Dichlorodihydrofluorescein diacetate (H_2DCFDA) dye. These authors have used 2,2'- azobis (2-amidinopropane) dihydrochloride (AAPH) as a source for peroxyl radicals. In our present experiment we studied the free radical scavenging activity of mussel hydrolysate on isolated mice lymphocyte cells.

Materials and method

Chemicals: Fetal Calf Serum (FCS), RPMI 1640, DCFDA, 2,2-Azo-bis (2-propionimidine dihydrochloride) (AAPH), Trypan blue, Ammonium Chloride, Sodium Chloride, EDTA, Tris HCl, Triton x-100. All chemical used were of highly purified grade.

Mussel hydrolysate of concentrations of 0.5 mg/ml, 1.0 mg/ml, 5 mg/ml and 10 mg/ml were screened for reactive oxygen species (ROS) scavenging activity using mice lymphocyte cells. Required concentrations of all these test samples were prepared in terms of weight by volume ratio in phosphate buffer solution.

Preparation of Reagents

(A) For Isolation of Lymphocyte:

❖ Phosphate Buffer Saline (10 mM, pH =7.4)

Composition: Na_2HPO_4 - 10 mM

NaH_2PO_4 - 10 mM

Saline (NaCl) - 0.84%

Preparation: 10 mM NaH_2PO_4, 10 mM Na_2HPO_4 and 0.84% saline were added to 800 ml of distilled water and pH was adjusted to 7.4. The final volume was made to 1000 ml.

❖ RPMI-1640 Media (for 1 liter):

Composition: $NaHCO_3$ – 3.7 g

Penicillin – 60 mg

Streptomycin – 250 mg

HEPES – 5.67 g

L-glutamine – 2.92 g

Preparation: 3.7 g $NaHCO_3$, 5.67 g HEPES, 2.92 g L-glutamine, 60 mg Penicillin, 250 mg Streptomycin (in the same serial order) were added to the 700 ml of autoclaved distilled water. This was followed by addition of RPMI-1640 powdered with constant stirring to avoid clump formation, pH was adjusted to 7.4 and the volume was made to1000 ml with autoclaved distilled water. The media was filtered and stored in a bottle at

4°C. The sterile condition was always maintained for preparing media to avoid contamination.

B: For RBC lysing buffer:

0.83% NH_4Cl was used for lysis of RBC cells.

C: For spleen cell counting stain

0.4% Trypan blue was used for counting spleen cells.

D: For Lysis buffer

Composition: 20 mM Tris-Hcl pH 7.4

2 mM EDTA

2 mM EGTA

0.1 mM Phenyl Methyl Sulphonyl Fluoride (PMSF)

1% Triton X 100

Protease inhibitor

Animal maintenance

Eight to ten week old BALB/c male mice weighing approximately 20-25 g were selected from inbred group maintained under standard conditions at temperature (25± 2° C) and humidity with 12 hour light and 12 hour dark cycles. The animals were fed with libitum feed. Usually four animals were housed in each sterile polypropylene cage containing a sterile paddy as bedding. All animal experiments were conducted with strict adherence to ethical guidelines laid down by the committee for the purpose of control and supervision of experiments on animals (CPCSEA) constituted by the Animal Welfare Division of the Government of India for the use of animals in scientific research.

Isolation of mice lymphocyte cells

Animals were killed humanely by cervical dislocation. Spleen was removed from the animals and kept in cold RPMI-1640 media (containing 15 mM HEPES, 2 mM L glutamine, 100 IU/ml pencillin, 100 mg/ml streptomycin and 20 μM 2-mercaptoethanol). Spleen was then gently crushed with the help of the crusher and centrifuged at 2000 rpm for 3 minutes. The supernatant was discarded and 3 ml of cold 0.83% NH_4Cl was added to the pellet and kept exactly for 3 minutes with proper mixing for the lysis of the RBCs cells. Cell suspension was diluted with RPMI-1640 media and centrifuged at 2000 rpm for 3 minutes. The supernatant was again discarded. The pellets were resuspended in

cold RPMI-1640 media and centrifuged at 2000 rpm for 3 minutes for two times. The supernatant was discarded and the pellets were resuspended in 5 ml of RPMI-1640 media and lymphocytes were isolated for further use.

Assay for carbon centered radicals by H_2DCFDA method

Mice lymphocytes at a density of $2x10^6$ cells/ml were incubated for one hour with mussel hydrolyzate of various concentrations. Cells were incubated with 20 mM dichlorofluorescin diacetate for fifteen minutes at 37^0 C before radiation. One set of cells was and treated with 10 mM AAPH [2, 2'- azobis- (2amidinopropane dihydrochloride)] for 20 minutes at 30° C whereas other set of cells was given a radiation dose of 2 Gy. Control set with only dichlorodihydrofluorescein diacetate (H_2DCFDA) was also kept for comparing the results. This was followed by determination of peroxyl radical concentration by measuring the increase in the fluorescence due to the oxidation of 2'7'-dichlorofluorescein (DCF) generated in the cells by flow cytometry on a Becton Dickenson FACS Vantage flow-cytometer. The mean fluorescence intensity at 530 nm was calculated using CellQuest software.

Results

There was a significant concentration dependent scavenging activity of free radicals with increasing concentration of mussel hydrolysate when an experiment was conducted on mice lymphocyte cells. These cells were irradiated by a radiation dose of 2 Gy in the present experiment. Free radical scavenging activity of mussel hydrolysate was increased from 48.52% at concentration of 0.1 mg/ml to 80.26% at a concentration of 10.0 mg/ml (Table 4.1). These results showed the higher scavenging of radiation induced ROS activity at the concentrations between 5.0 and 10 mg/ml of mussel hydrolysate. In control cells the free radical scavenging activity was negligible (Table 4.1). This indicated that mussel hydrolysate possess a high degree of scavenging of radiation induced ROS ability in a dose dependent manner (Table 4.1; Fig. 4.1).

Similarly when free radicals were induced chemically by Azo-bis, there was also a concentration dependent scavenging of free radicals with increasing concentration of mussel hydrolysate. Maximum scavenging activity was observed at a concentration of 10 mg/ml. Free radical scavenging activity of mussel hydrolysate increased from 51.12 to 80.77% with mussel hydrolysate concentrations from 0.1 mg/ml to 10 mg/ml (Table 4.2). In control cells, free radical scavenging activity was found to be negligible (Table 4.2; Fig. 4.2).

In both the experiments, mussel hydrolysate showed significant free radical scavenging activity on chemically and radiation induced free radicals formation. At 0.1 mg/ml of mussel hydrolyzate, the scavenging activity was 48.52% when a radiation dose of 2 Gy was given. However, scavenging activity was 51.12% when ROS was induced chemically by Azo-bis. Similarly, the scavenging activity was increased to 80.26% at a concentration of 10 mg/ml of mussel hydrolysate at 2 Gy dose of radiation as compared to 80.77% with Azo-bis. Not much variation of free radical scavenging activity was observed at both the concentrations of 0.1 mg/ml and 10 mg/ml of mussel hydrolysate using radiation induced ROS and chemically induced by Azo-bis as evident from Tables 4.1 & 4.2.

Discussion

Ionizing radiation and certain cytotoxic drugs are known to induce oxidative stress through generation of Reactive Oxygen Species (ROS). This causes imbalance of the pro-oxidant and anti-oxidant state of the cells, which finally results to cell death (Lin *et al.,* 1995; Aoshima *et al.,* 1997). Intracellular generation and accumulation of ROS such as superoxide, hydrogen peroxide, singlet oxygen, hydroxyl radical and peroxyl radical in the stressed cells overcomes the natural antioxidant defense causing damage to biological macromolecules like nucleic acids, proteins and lipids (Aoshima *et al.,* 1997). The induced free radicals caused lipid peroxidation in the membrane of cells reflected in altered physio-chemical properties in lipid bilayer and eventually led to cellular toxicity (Pandey and Mishra, 1999; 2002; 2004; Berroud *et al.,* 1996; Schmidt *et al.,* 2000; Marathe and Mishra, 2002). The potential application of radioprotective chemicals in the event of radiation accidents/incidents has been investigated from the beginning of the nuclear era (Weiss and Simic, 1988). It has also been considered that the side effects of radiation therapy for cancer patients could be controlled by the use of radioprotectors by the protection of normal tissue. Majority of the antioxidants studied as radioprotectors contain sulfhydryl groups (Weiss, 1997). Any substance, scavenging the free radicals can be used as radioprotectors provided that it is not having any toxic side effects.

Ionizing radiations like gamma rays and U.V rays are also known to induce oxidative stress through generation of reactive oxygen species resulting in imbalance of the pro-oxidant and antioxidant state of the cells which is suggested to culminate in cell death (Lin *et al.,* 1995; Aoshima *et al.,* 1997). Ionizing radiation causes damage to living cells / organisms essentially due to direct and indirect actions (Lea, 1955; Altman, *et al.,* 1970). Radiation can produce biological damage indirectly through the radicals generated by radiolysis of water and known as Indirect Effect (Indirect action) of radiation (Alaoui *et al.,* 1992). Free radicals like (free H°, OH°, HO_2 and H_2O_2) are formed within microseconds after irradiation of aqueous solution. Indirect Effect is the major cause of biological damage by low linear energy transfer radiation like gamma

rays while direct effect is dominant with high linear energy transfer radiation like alpha particles and high-energy neutrons. More than 70% of molecular damage caused by low linear energy radiation is attributed to the indirect effect mediated through the free radicals.

In addition to antioxidant enzymes there are substances, which are used as radioprotectors by means of their free radical scavenging activity. The mechanism of free radical scavenging suggested that certain agents are oxidized by free radicals, forming stable compounds incapable of reacting with other cellular components. This mechanism prevents the free radicals from reacting with the cell vital components (Varanda and Tavares, 1998). Additionally, these substances scavenge free radicals before they can cause damage to important macromolecules like DNA, proteins and lipids. Several free radical scavengers are known to interact with aqueous free radicals that in turn prevent the radiation induced lethality of cells. These free radical scavengers include sulphydryl compounds, vitamins, hormones, nitroxides, angiotensin converting enzyme inhibitors, plant extracts and isolated compounds (Nair *et al*, 2001). Sulfhydryl compounds like cysteine, cysteamine, glutatathione, WR 2721 and other WR compounds render radioprotective mechanism by acting as a free radical scavenger.

In the present study, the radioprotective activity of mussel hydrolysate was screened by studying the free radical scavenging activity by means of DCHFDA (2'-7 Dichlorodihydrofluorescein diacetate) as fluorescence probe. There was a concentration dependent increase in the free radical scavenging activity of mussel hydrolysate on both chemically induced and radiation induced free radical formation. Free radical scavenging activity increased from 48 to 80% with increasing concentration of extract from 0.1 mg/ml to 10 mg/ml. Mussel hydrolysate showed a high free radical scavenging activity when lymphocyte cells were pre-incubated with extract for 30 minutes. There was a slight toxicity at a concentration of 10 mg/ml. Santhosh *et al.* (2004) have showed that chlorophyllin at concentration of 1.0 μM decreased significantly the formation of ROS level in mice lymphocytes and further it was found to decrease at higher concentrations of chlorophyllin.

Sushama *et al* (2005) have studied the radiosensitizing effect of elagic acid on tumor cell lines and found that 100 μM/L of this compound increased the ROS significantly after irradiation dose of 2 Gy. Pandey and Mishra (2004) found that eugenol, a plant product at a concentration of 100 μM, significantly decreased the ROS formation in thymocyte cells after irradiation with 0.5-200 Gy. The present results also showed that mussel hydrolysate decreased the formation of ROS significantly after irradiating the lymphocyte cells by a dose of 2 Gy. Free radical scavenging activity of mussel hydrolysate could be because of the complex mixture of various amino acids and metal ions present in the extract (Rao *et al.*, 2002). Among the various aminoacids, cysteine has been reported to be the earliest known radioprotector and act as a free radical scavenger (Pandey and Mishra, 2004). The mussel hydrolysate (100 ml) has been reported to contain nearly 0.05 g of cystein (Boikov *et al.*, 1997). So the free radical scavenging activity of mussel hydrolysate could be due to the presence of this particular amino acid.

Several aliphatic alcohols including ethanol, ethylene glycol, glycerol etc are found to be good radical scavengers. Metodiewa *et al.* (1996) have reported that two compounds Tempace and Troxyl, which are 2,2,6,6- tetramethyl piperdine derivatives, act as scavengers of superoxide, inhibitors of iron and ascorbate driven by Fenton reaction. Melatonin (N-acetyl-5-methyloxytryptamine), a pineal gland hormone involved in regulating the neuroendocrine axis, is highly efficient scavenger of hydroxyl and peroxyl radicals besides peroxynitrite. It has also been reported to be an important antioxidant compound (Pieri *et al.*, 1994; Pierrefiche and Laborit, 1995; Reiter, 1995; Vijayalaxmi *et al.*, 1996). Antioxidants like vitamins C and E offer radiation protection because radiation damages mimic the oxidative stress associated with active oxygen toxicity (Wilson *et al.*, 1983). The radioprotective effects of ascorbate seem to be due to its interactions with radiation-induced free radicals (Duschense *et al.*, 1975). Vitamin E, a singlet oxygen scavenger, does not scavenge hydroxyl radicals or hydrogen peroxide. A water-soluble vitamin E, derivative, TMG (tocopherol monoglucoside) scavenges free radicals and effectively protects DNA and membranes against ionizing radiation (Kapoor *et al.*, 2002; Rajagopalan *et al.*, 2002).

Pandey and Mishra (2004) have studied the generation of reactive oxygen species (ROS) and cellular apoptosis and also their modification by a natural antioxidant obtained from plant – eugenol using mouse thymocytes. Sandhya and Mishra (2005) have studied the cytotoxic response of breast cancer cell lines MCF-7 and T-47D using triphala - an Indian ayurvedic formulation with anticancer properties. They found that triphala posses anticancer properties as it produced reactive oxygen species in cancer cells leading to apoptosis. The free radical formation in these cancer cell lines was measured by Sandhya and Mishra (2003) by DCF-FDA fluorescent probe.

Santhosh Kumar *et al.* (2004) have studied the effect of chlorophyllin against oxidative stress in splenic lymphocytes both for *in vitro* and *in vivo* studies where H_2DCFDA dye and AAPH were used as a source of peroxyl radicals. Sushama *et al.* (2005) have studied the radiation induced oxidative stress and cytotoxicity in tumor cells by ellagic acid. Reactive oxygen species level was measured by spectrofluorimetric method by using 2'-7 Dichlorodihydrofluorescein diacetate (DCHFDA) fluorescent probe. Bhavani *et al.* (2003) have studied the formation of reactive oxygen species and radiation response in mice lymphocytes and tumor cells by using DCHFDA fluorescent probe. Girdhani *et al.* (2005) have studied the cytotoxic effect of anticancer and antioxidant agents namely; Eugenol, (EU), Ellagic acid (EA), Triphala (TPL), Tocopherol Succinate (TOS) and Arachidonic acid on normal and cancer cells by measuring oxidative damage, membrane alteration and damage to nucleic acid. These investigators have measured the ROS generation by DCF-FDA fluorescent probes after exposure to low to moderate doses of gamma radiation.

In the present investigation, the protection of whole body irradiation by mussel hydrolyzate was studied for i*n vivo* experiments. Intraperitonial administration of mussel hydrolysate (100 µg) daily for 5 days followed by the exposure of animals to γ-radiation (500 rad) showed significant protection of mice when the experiments were performed using GM-CSF colony forming assay (Parab and Chatterji US Patent No, 7,087,429; 8 July' 2006). Unlike most of the natural products presently under evaluation for radioprotective property, mussel hydrolyzate is known to yield small and stable molecules with very low structural complexity, which showed the presence of antiviral, antibacterial and antiparasitic activities (Chatterji *et al*, 2002).

The ionizing radiation exposure to human has been increasing day by day that includes medical X-ray, CT scans and also nuclear accidents and nuclear terrorism (Wilson, 2000). Under these circumstances, a non-toxic extract from natural source could be an ideal radioprotector useful in preventing diseases like cancer and degenerative diseases arising from gene aberrations. The mussel hydrolysate used in the present study possesses free radical scavenging activity and could prevent the damages induced by ionizing radiations. Further studies will be required to find out the actual mechanism of action or active ingredient of hydrolysate from *Perna viridis* to develop a potential radioprotector for its use in cancer therapy.

Table 4.1: Scavenging of radiation induced ROS by mussel hydrolysate

Test sample	Percentage of ROS formation by 2 Gy dose				Mean	S.D (±)	Free radical scavenging activity (%)
	Expt 1	Expt 2	Expt 3	Expt 4			
Control irradiated	100	100	100	100	100		
Extract 0.1 mg/ml	58.81	43.71	52.85	50.58	51.48	6.2	48.52
Extract 0.5 mg/ml	44.11	40.15	43.22	42.18	42.41	1.7	57.59
Extract 5.0 mg/ml	26.23	24.82	25.77	25.13	25.48	0.6	74.52
Extract 10.0 mg/ml	20.35	17.35	20.82	20.45	19.74	1.6	80.26

Table 4.2: Scavenging of chemically induced ROS by mussel hydrolysate

Test sample	Percentage of ROS formation by Azo-Bis				Mean	S.D (±)	Free radical scavenging activity (%)
	Expt 1	Expt 2	Expt 3	Expt 4			
Control irradiated	100	100	100	100	100		
Extract 0.1 mg/ml	49.37	48.76	48.87	48.54	48.88	0.35	51.12
Extract 0.5 mg/ml	30.23	36.58	36.90	36.65	35.09	3.24	64.91
Extract 5.0 mg/ml	24.36	23.55	23.86	23.60	23.84	0.37	76.16
Extract 10.0 mg/ml	17.88	19.80	19.68	19.58	19.23	0.90	80.77

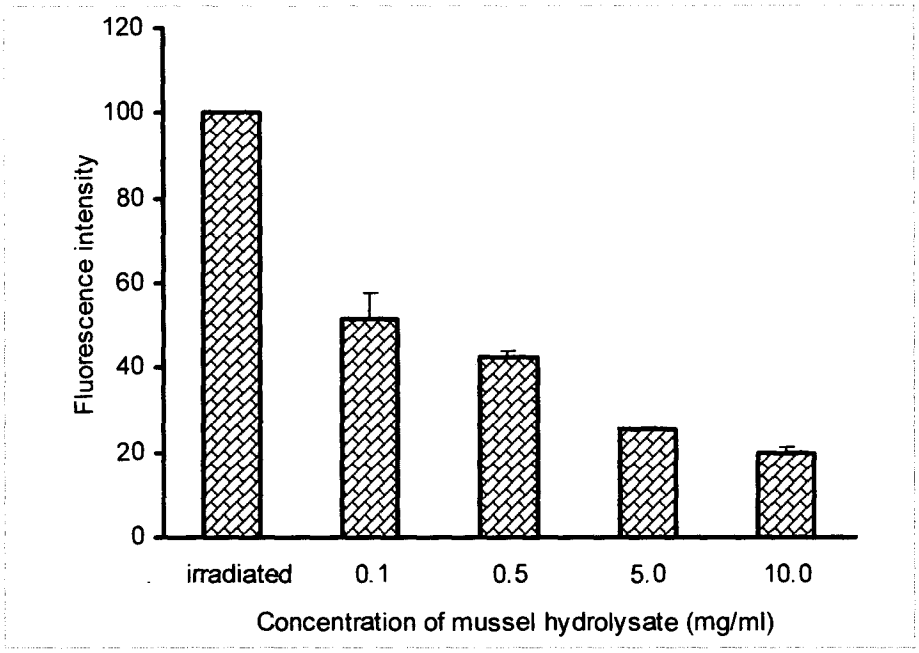


Fig. 4.1 Scavenging of radiation induced ROS by mussel hydrolysate

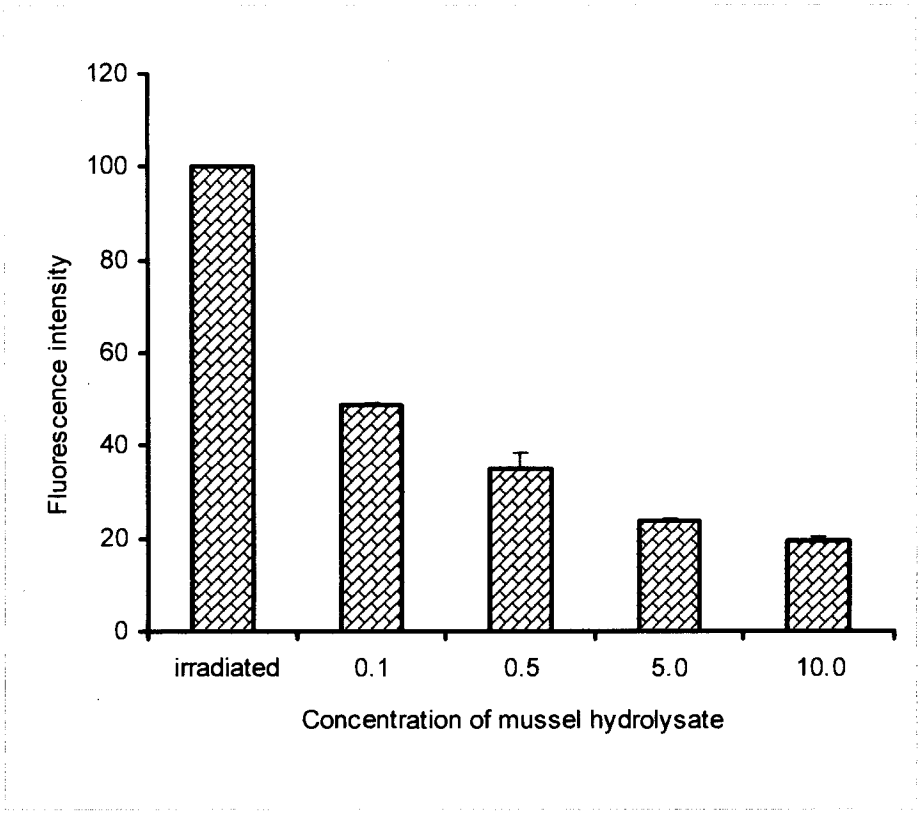


Fig. 4.2: Scavenging of chemically induced ROS by mussel hydrolysate

CHAPTER 5

Protection of DNA damages by ionizing radiation using mussel hydrolysate

Matter is made up of tiny units called atoms. Every atom has a nucleus and a surrounding cloud of electrons. The nuclei of some atoms, like those of K^{40}, are unstable as compared to the atoms, which spontaneously change their structure and consequently their physical and chemical properties. When an unstable nucleus undergoes changes, invisible particles or waves are released. The particles or waves are called radiation and the unstable nucleus is said to be radioactive. Radioactive nuclei are called radionuclides. Hall (2000) described this, as "*Radiation is energy in the form of particles or waves which has been present since the beginning of the universe*".

The process of ionization is important in understanding the radiation because it is the process that differentiated ionizing radiations from the other types. Ionization basically is a process of removing electrons from atom. If enough energy is supplied to remove electrons from the atom, the remaining has a positive charge. The positively charged atom and negatively charged electrons are called as "ion pair". Ionization and radiation are two different processes. Ions (ion pairs) can be the result of radiation exposure and allow the detection of radiation. Radiation is ionizing if it disrupts the chemical bonds of molecules of which living things are made and thus cause biologically important changes. When ionizing radiation passes through matter, they dissipate their energy in path through the ejection of electrons from the outer shell of atoms, which results in the loss of atomic balance. Negatively charged particle (electron) leaves atoms which are no longer neutral but are positively charged. The positively charged atom is called as an ion. The ejected electrons are free from their respective atoms and when their energy dissipated, they get themselves attached to other atoms and converted the ion into negatively charged ions. To achieve their stable configuration (e.g. neutral charge), ions undergo many chemical reactions and during these reactions ionizing radiation is thought to cause biological damage.

The basic types of Ionizing radiation of primary concern to us are namely; alpha particle, beta particle, gamma rays, X-rays, neutron particles and other fast moving particles like protons, electrons and heavy ions. Gamma rays produce ionizing effects in biological tissue through Compton Scattering Process, photoelectric effects and to a lesser extent, through pair production. β-particle, α-particle and positrons ejected orbital electrons from the atom and thus ionization reaction occurs. Gamma rays, alpha

particles and protons are emitted from atomic nuclei by the interaction of neutron with matter producing ionizing effects on tissues (Hall, 2000). Ionizing radiation has been reported to cause damage to living cells / organisms essentially due to direct & Indirect actions (Altman *et al.*, 1970).

Direct effect of radiation: Radiation can directly be interacted with the critical target molecules in the cell causing ionization and excitation and which in turn lead to biochemical lesion. This is known as *direct effect (direct action) of radiation.*

$$RH \xrightarrow{\text{radiation}} RH^{+}$$

$$RH^{+} \longrightarrow R^{\circ} + H^{+}$$

Direct action of radiation on biomolecules results in the ejection of orbital electron from their atoms. Electron ejection takes place in less than one second. Ejection of valence electron from their orbits breaks covalent bonds and cleavage of biomolecules. Ejection of other orbital electron ionizes the molecules by breaking or changing non covalent bonds and also altering its molecular conformation. In both the cases the biological activity of the molecule is altered or destroyed.

Effect of radiation on water molecule

a) Ejection of orbital electrons from H_2O ionizes into H^+ and the highly oxidizing free hydroxyl (OH°) radical:

$$H_2O \rightarrow H_2O^{+} + e^{-}$$

$$H_2O^{+} \rightarrow H^{+} + OH^{\circ}$$

The above-mentioned reactions are completed in 10^{-9} seconds and lead to further reactions (b) within a few microseconds.

b) After losing most of their kinetic energy, β-particles and ejected electrons may be absorbed by H_2O which consequently ionized into OH^- and the highly reducing free hydrogen (H°) radical as follows:

$$H_2O + \beta^{-} \rightarrow H_2O^{-}$$

$$H_2O^{-} \rightarrow H^{\circ} + OH^{-}$$

The above chain like reactions is completed in 10^{-9} second and further lead to the following reactions within a few microseconds as described below:

i) Some free hydroxyl radicals react with each other to produce H_2O_2, which is a very strong oxidizing agent

$$OH^\circ + OH^\circ \rightarrow H_2O_2$$

ii) Free hydrogen radical either reacts with molecular O_2 to form highly oxidizing hydroperoxyl ($.HO_2$) radical, or reduces some H_2O_2 to H_2O, or joins hydrogen radical to form H_2 as follows.

$$H^\circ + O_2 \rightarrow H_2O^\circ$$

$$2\ H^\circ + H_2O_2 \rightarrow 2\ H_2O$$

$$H^\circ + H^\circ \rightarrow H_2$$

iii) The hydroperoxyl radical may join free hydrogen radical to form H_2O_2

$$HO_2^\circ + H^\circ \rightarrow H_2O_2$$

Free radical and H_2O_2 thus formed by the direct action of radiation on H_2O may in turn affect other biomolecules.

Indirect effect of radiation: Radiation can produce biological damages indirectly through the radicals generated by radiolysis of water. This is called the *Indirect Effect (Indirect action)* of radiation (Alaoui *et al.*, 1992). Free radicals like (free H°, OH°, HO_2 and H_2O_2) are formed within microseconds after irradiation of aqueous solution. The aqueous free radical OH° and H° interact with the target molecule in their immediate vicinity to form organic free radicals as follows:

$$RH + OH^\circ \longrightarrow R^\circ + H_2O$$

$$RH + H^\circ \longrightarrow R^\circ + H_2$$

Hydrated electrons can also interact with target molecule producing free radicals. These organic free radicals can combine with molecular oxygen to produce organic peroxides leading to permanent damage.

$$R^\circ + O_2 \longrightarrow ROO^-$$

Indirect effect is the major cause of biological damage by low LET (Linear Energy Transfer) radiation like γ-rays. Additionally, the direct effect is dominant with high LET radiation like alpha particles and high-energy neutrons. More than 70% of molecular damage caused by low LET radiation is attributed to the indirect effect mediated through the free radicals.

Effect on genetic material: Ionizing radiation is a well-established carcinogen causing oxidative damage (Toule, 1987; Ames and Gold, 1991; Halliwell and Aruoma, 1991). DNA is the principal cellular target of Ionizing radiation. It causes damage to DNA by both direct and indirect ways. Charged particles and photons directly eject electron from the DNA molecules and subsequently ionize them. This is the direct effect of ionizing radiation (Steenken, 1989). In biological process, genetic mutation is on account of direct action of ionizing radiation on chromosomal DNA. On the other hand, fast moving electrons ejected from other molecules may indirectly ionize DNA (Toule, 1987). Indirectly, ionizing radiation reacts with surrounding water molecules and produces free radicals like superoxide radical, hydroxyl radical etc. A wide variety of biochemical consequences of radiation-induced damage to DNA occur because of free radical attack (Breen and Murphy, 1995). In both the cases, ionizing radiation causes different types of lesions in the DNA molecules. These lesions include base damage, strand breaks and cross-links.

1. Base damage

Bases can change into different compounds by a variety of chemical and physical agents. Damage to base residues can rupture N-glycosidic bond and formed a basic deoxyribose. Altered bases may represent alkali labile sites or AP sites. For instance ionizing radiation (such as the β and particles emitted by naturally occurring radioisotopes or X-ray) can break purines and pyrimidines ring that produces about 30 modified bases and sugars in DNA. Among nucleic acid bases, guanine is the most susceptible DNA target for oxidative reactions because of its lowest ionization potential. This reaction is mediated by $OH^{\cdot}$ and other free radicals (Cadet *et al.*, 1999). Thus, one of the mutagenic and the most abundant lesions formed in irradiated chromatin is the 8-hydroxyguanine (Kasai *et al.*, 1986). The most common and abundant measurable oxidative DNA base adduct is 8 hydroxy deoxyguanine (8-OHdG) and is considered to be a key biomarker related to carcinogenesis (Floyd, 1990). Some of the modified bases are listed below:

1. 8-hydroxyguanine
2. 8- hydroxy deoxyguanine (8-OHdG)
3. Cis and trans thymine glycol
4. 5-hydroxy-5-methylhydantoin
5. 5-hydroxycytosine
6. 5,6-dihydroxy-5, 6-dihydrouracil (Uracil glycol)
7. 4,6-diamino-5-formamino pyrimidines
8. 7,8-dihydro-8-oxoadenine
9. 8,5'-cyclodeoxyaddenosine
10. 2,4-diamino-5-formamidopyrimidine-6-one

2. DNA strand breaks

2.a: DNA single strand breaks

The formation of single strand break generally occurred when a cell is exposed to radiation. This single strand break is at least 20 times more than those of double strand breaks (Elia *et al.,* 1991). Single strand breaks are due to breaking of DNA chain, non-enzymatically or enzymatically. It is due to the interaction of free radicals with sugar moieties led to the clevagae of the sugar-phosphate backbone of DNA followed by single strand breaks (Toule, 1987).

2.b: DNA double strand breaks

When a DNA molecule has sufficiently large number of randomly located single stranded breaks and two breaks are situated opposite to each other, then it cause the breakage of the double helix. Double strand breaks show more serious consequences as they are well correlated with the cytotoxic effects of ionizing radiation and considered the primary lesion involved in cellular death (Elia *et al.,* 1991).

The DNA repair mechanisms induced after exposure to ionizing radiation is inefficient, the damaged DNA strands that are copied during replication, led to mutagenesis and carcinogenesis (Halliwell and Aruoma, 1991). The single strand break may be rectified by DNA repair enzyme within a few minutes. However, a double strand break escapes repair and often results in mutation, chromosomal aberration, cellular death or malignancy (Sankaranarayanan, 1999). Damage to chromosomes may retard or stop DNA replication and consequently retard the mitotic rates resulting in structural and functional abnormalities. The continuation of DNA replication even after radiation

damage resulted in chromosomal fragmentation, sticky chromosomal bridges, dicentric chromosomes, polyploidy, pyknoic or hyper-segmented nuclei and neoplasm (Lea, 1955). These processes are associated with an increased risk for numerous genetically determined diseases (Sankaranarayanan, 1999).

Protection of DNA by radioprotectors

DNA is the principal target of radiation damage and the compounds having ability in preventing damages to DNA are more important in cancer research. There are a number of compounds, which showed radioprotective ability by preventing damages to DNA. Most of them protect the DNA by scavenging the free radicals especially the hydroxyl ($OH^{\cdot}$) radicals before they cause damage to DNA. A number of antioxidants such as Sulfhydryl compounds (disulfiram at 100 and 200 μM) reduced glutathione (10 and 100 mM), purified compounds of plant origin, such as curcumin (1 mM, 10 mM and 100 mM), quercetin (100 and 500 μM), rutin (100 and 200 μM), ellagic acid (100, 200, and 500 μM) and gallic acid (100 and 500 μM) have been tested for their efficacy in radioprotection using yeast cells (Nemavarkar *et al.,* 2004). All these compounds except gallic acid are specifically protected normal yeast cells from gamma-radiation damage. Using rad52 mutants, which lacks recombinational DNA repair pathway, has been found to considerably to reduce the DNA damage rather than repairing the DNA (Nemavarkar, *et al.,* 2004).

It has been found that caffeine showed radioprotection by reducing DNA damage in yeast cells (Purva and Pasupathy, 2001). Water-soluble vitamin E at a concentration of 1-1.5 g/kg body weight showed to protected DNA damages in mice on *in vivo* studies (Nair *et al.,* 2003). Disulfiram, which is used for aversion therapy in alcoholism, protect DNA and breaking of single strand breaks in plasmid at a concentration of 50 mg/kg body weight in mice (Gandhi *et al.,* 2003). Rajagopalan *et al.* (2003) have found that vinblastine, a natural alkaloid from the plant *Vinca rosea* protected DNA in mice lymphocyte cells whereas, Maurya *et al.* (2004) found that the Glycyrrhizic acid, a root extract of the plant *Glycyrrhiza glabra,* showed radioprotection of plasmid DNA and also leukocyte cells. Troxerutin has also been reported to protect DNA in mice *in vivo* studies (Maurya *et al.,* 2004). In another study Huo *et al.* (2004) have showed that enzymatic extracts from brown seaweeds, protected lymphocyte DNA *in vitro.* Gandhi and Nair

(2005) found that extract of plant *Terminalia chebula* protected plasmid DNA as well as mice leukocyte DNA.

DNA damages can be studied by various methods such as micronucleus assay, single strand break formation, conversion of super coiled form to open circular form in plasmid pBR322 and also by Single Cell Gel Electrophoresis (SCGE) or Comet assay. The conversion of supercoiled form into open circular form during DNA exposure to radiation causes difference in the mobility in the agarose gel because of the formation of the strand breaks in the DNA (Gandhi *et al.*, 2004).

The comet assay or single cell gel electrophoresis (SCGE) is commonly used assay for quantifying and analyzing DNA damages in individual cells. Ostling and Johansson (1984) were the first to develop the technique of comet assay. Singh (2000) modified this technique as alkaline comet assay. In this assay, cells embedded in agarose on a microscope slide are lysed with detergent and high salt to form nucleoids containing supercoiled loops of DNA which is linked to the nuclear matrix (Collins, 2004).

Electrophoresis at a high pH results in a structure that resembles comets having head and tail regions, which can be observed by fluorescence microscopy. The head is composed of intact DNA and tail region consisted of damaged (single- strand or double-strand breaks) or broken pieces of DNA. The intensity of the comet tail relative to the head reflects the number of DNA breaks. The basis for this is that loops containing a break lose their supercoiling and become free to extend toward the anode. The assay has applications in testing novel chemicals for genotoxicity, monitoring environmental contamination with genotoxins, human biomonitoring and molecular epidemology and also for fundamental research in DNA damage and repair (Collins, 2004). DNA repair can be monitored by incubating cells after treatment with damaging agent and measuring the damage that remained at a particular interval.

MATERIALS AND METHOD

The materials such as Plasmid pBR322 (Bangalore Genei) and Ethidium Bromide (Sigma) were obtained from the market whereas Tris acetate buffer (pH 8.3) (1X diluted from a 100X stock) was prepared in the laboratory

Protocol

The induction of strand breaks in plasmid pBR322 DNA *in vitro* by γ-radiation was studied using agarose gel electrophoresis method as described by Gandhi *et al.* (2004) and Maurya *et al.* (2005). Plasmid pBR322 DNA (250-300 ng) dissolved in 10 µl of 100 mM sodium phosphate buffer (pH 7.4) after incubating it with mussel hydrolyzate at a concentration of 25 mg/ml and then it was exposed to various doses of γ-radiation (30-360 Gy). This was done at dose rate of 6.0 Gy/min on ice using a Gamma cell 220 (Atomic Energy Canada Ltd, Ottawa, Canada). In another experiment, plasmid DNA was incubated with increasing concentrations of mussel hydrolyzate (5-50 mg/ml) and irradiated with radiation dose of 120 Gy. The supercoiled (ccc) and open circular (oc) forms of DNA were separated by agarose gel electrophoresis using 1% agarose gel in 100 mM Tris acetate, 2 mM EDTA buffer of pH 8.3 (Sambrook *et al.*, 1989).

DNA bands after staining with ethidium bromide were photographed using a Genius gel documentation system. The images of DNA bands were analysed using software provided along with the system. Radiation induced DNA damage was estimated as an increase in open circular (oc) form of DNA. All irradiation mixtures of mussel hydrolysate and DNA were prepared in buffer in required concentrations just prior to irradiation. The percentage oc and ccc form of each DNA sample was calculated from the total intensities of the oc and ccc bands {[oc]/[ccc]+{[oc]}x100. Further, all values were compared as percentage of ccc in unirradiated control, [(% ccc of test) / (ccc of control)] x100 (Gandhi and Nair, 2005).

Reagents for Comet assay

1) **Lysing Buffer:** The lysing buffer was prepared with 2.5 M NACl; 100 mM Na_2EDTA; 10 mM Tris HCl, 1% Triton X100, 1% DMSO, 1% Sodium Sarcocinate or Lauryl Sarcocinate of pH 10.

2) **Electrophoresis Buffer:** The electrophoresis buffer was prepared with 300 mM NAOH; 1mM Na_2EDTA; 0.2% DMSO and pH were kept more than 13.0.

3) **Neutralising Buffer:** The neutralising buffer was prepared with 0.4 M Tris-HCL of pH 7.4.

Single–cell gel electrophoresis (Comet assay)

The DNA breaks in mice lymphocyte were measured by the use of single–cell gel electrophoresis (Comet Assay) based on the method of Singh (2000) with minor modifications. Fully frosted microscope slides (Gold Coin, Mumbai) were covered with 200 µl of 1% normal melting agarose (NMA) in PBS at 45^0 C and immediately cover-slipped. This slide was kept at 4^0 C for 10 minutes to allow the agarose to solidify. The removal of the cover slip from agarose layer was followed by an addition of a second layer of 200 µl of 0.5% low-melting agarose (LMA) containing approximately $2x10^4$ cells at 37^0 C. Cover slips were placed immediately and the slides were placed at 4^0 C. After solidification of the LMA, the cover slips were placed in chilled lysing solution (2.5 mM NaCl, 100 mM EDTA, 10 mM Tris-HCl, pH,10, 1% Triton-X100, and 1% sodium lauryl sarcosine) for 1 hour at 4^0 C. The slides were then placed into an electrophoresis tank filled with freshly prepared alkaline buffer (300 mM NaOH, 1.0 mM Na_2-EDTA and 0.2% DMSO, pH> 13.0) for 20 minutes for DNA unwinding.

For electrophoresis of the DNA, an electric current of 40 V / 350 mA for 20 minutes at 4^0 C was used. After electrophoresis, the slides were washed gently with neutralizing buffer (0.4 M Tris-HCl buffer, pH 7.4) to remove the alkali. The slides were stained with ethidium bromide and visualized under a fluorescent microscope (Carl Ziess Axioskop) with bright field phase contrast and epifluorescence facility attached to a high-

performance JVG Tk 1280 camera. The integral frame grabber used in this system (Cvfbolp) was a PC-based card, which accepted color composite video out put of the camera. For this study, about 50 cells/slide were captured by the camera. The quantification of the DNA strand breaks of the stored images was done with CASP software by which tail length, tail moment and percent DNA in the tail, olive tail moment were directly obtained. Calculation of these three parameters included in the software and the extent of DNA damage was analysed using these three parameters.

RESULTS

In the present study, the mussel hydrolysate showed significant protection of plasmid DNA from γ-radiation induced strand breaks in *in vitro* experiment. Mussel hydrolysate (25 mg/ml) when used in the present experiment, showed a significant protection of plasmid DNA from damages generated upon exposure with increasing doses of γ-radiation from 30 Gy to 360 Gy (Figs 5.1 and 5.2). In this study, when the mussel hydrolysate in the concentration of 5 mg/ml to 50 mg/ml was added 30 minutes prior to radiation, there was significant reduction in the disappearance of **ccc** form of DNA in a concentration dependent manner (Figs. 5.1 and 5.2). However, an exposure of 120 Gy γ-radiation was found to reduce the **ccc** levels approximately up to 19% as compared to un-irradiated control (100%) that indicated a significant reduction of **ccc** form due to strand breaks. In comparison with irradiated control, the presence of 25 mg/ml mussel hydrolysate during radiation was found to restore the **ccc** levels up to 81% whereas 50 mg/ml mussel hydrolysate restored the **ccc** levels up to 100% (Figs. 5.3 and 5.4).

The photomicrographs of comet assay as presented in Figure 5.5, showed a significant reduction in DNA damage with 0.5 mg/ml mussel hydrolysate in mice lymphocytes when exposed to gamma irradiation of 2 Gy. The morphology of the lymphocytes cells were remained same in control and cells treated with mussel hydrolysate. However, the cells treated with 2 Gy radiations showed a clear deformity of the lymphocyte cells indicating the damaging effect by the radiation.

Frequency distribution histograms of tail length, tail moment and percent DNA in tail of the control, control irradiated and cells treated with mussel hydrolysate are given in Fig. 5.6. It is conspicuous from this figure that the number of cells with higher values for these three parameters increased with respect to those of un-irradiated control when the cells were exposed to a gamma radiation dose of 2 Gy. However, when the cells were irradiated in presence of 0.5 mg/ml mussel hydrolysate, the number of cells having

higher values of tail length, tail moment and percent DNA in tail decreased as compared to irradiated cells.

Similarly, TDNA in tail was increased from 4.397±1.2 μm to 35.61±1.98 μm, tail length from 11.36±0.35 μm to 63.08±2.89 μm, tail moment from 1.419±0.231 μm to 26.03±1.235 μm and olive tail moment from 1.731±0.012 to 6.141 ±1.255 μm when cells were irradiated by 2 Gy radiation dose. But the prior incubation of cells with mussel hydrolysate for 30 minutes brought these parameters down to the levels of 13.43±1.23 μm, 29.91±1.9 μm, 4.72±0.921μm and 2.436±1.078 for TDNA, tail length and tail moment and olive tail moment respectively (Figs 5.7 a & b). These results clearly indicate that the mussel hydrolysate significantly reduced the DNA damages in lymphocyte cells which was induced by gamma radiation.

DISCUSSION

The radiation damage to a cell depends on several factors like presence of oxygen, sulfhydryl compound and other molecules in the cellular milieu (Haynes *et al.*, 1975). Normally during radiotherapy high doses of radiation are recommended to the tumor site in order to kill as many cancer cells as possible and still allowing the normal cells to repair and recover (Nair *et al.*, 2001). This is clinically not feasible, since the normal tissues surrounding the tumor are well profused, vascularised and remained oxygenated and therefore they are more prone to radiation damage (Dave *et al.*, 1991). In radiation therapy, high-energy beams of radiation are focused on the tumor site from which cancerous cells were removed. Radiation works by causing damage or changes to the cells in the tumor site. However, overdose of radiation, damages both normal as well as cancer cells and in order to obtain better tumor control with a higher dose; the normal tissues should be protected against radiation injury. Thus the role of radioprotective compounds has become very important in clinical radiotherapy.

During radiation therapy, cellular injury occurs through generation of free radicals primarily targeting the DNA (Toule, 1987; Ward *et al*, 1993; Ames and Gold, 1991). The nuclear region of the cell containing DNA is 100 times sensitive than cytoplasm. Highly reactive oxygen radicals produced by ionizing radiation causes lesions in DNA that lead to mutations and cell death. The damage to DNA includes single strand or double strand breakages which in turn lead to delay in cell division, formation of modified cell, neoplastic formation, point mutation that causes chromosomal aberration, cell loss and ultimately the cell death (Sonntag, 1987). Normally, single strand breaks are repaired by DNA polymerase enzymes. However, when these damages are continued and accumulated considerably, it causes cancer (Halliwell and Aruoma, 1991). Thus any compound capable of reducing the generation of free radical and thereby preventing the damages to DNA is useful as a radio protector (Chevion *et al.*, 1999).

Native plasmid is predominantly remained in supercoiled form (ccc) and when exposed to γ-radiation it suffered strand breakage and converted into open circular form (oc). The reduction in the quantity of the supercoiled form (ccc) of plasmid DNA into open form is directly related to the radiation-induced damage particularly strand breaks in DNA. Both the supercoiled and open forms of DNA are separated with the help of agarose gel electrophoresis to compare the DNA damages which can further be studied by ethidium bromide staining and visualized under the UV light as shown in Figure 1. In present experiment, the protection of plasmid showed the DNA protecting ability of the mussel hydrolysate in an *in vitro* system. In another study a similar observation has been made that showed the prevention of plasmid DNA damages by using commercially available disulfiram (Gandhi *et al.*, 2003).

The technique of single gel electrophoresis (comet assay) has been reported to be a rapid, sensitive and visual method to quantify DNA strand breaks in an individual cell (Rajagopalan *et al.*, 2003). Hence in the present study, an *ex vivo* protection of DNA was studied by means of comet assay using mice lymphocyte cells. The assay showed lower values of number of cells primarily for three parameters *viz.* tail length, tail moment and percent DNA in the tail, when lymphocyte cells were treated with mussel hydrolysate. The DNA damage occurred after exposing to a radiation dose of 2 Gy was sufficient to form long tail for the comets. The tail length of the comet indicated the extent of damage since the smaller molecules moved faster on the agarose gel. The longer tails of the comets also indicated that the strand breaks were more frequent and the DNA was fragmented to several small molecules. Percent DNA in the tail gave the percentage of the DNA in the tail of the comet taking the total DNA of the cell as 100%. Tail moment is a commonly accepted unit of DNA damage, which normalized the difference in the size of the nucleus. It is the product of fraction of the DNA in the tail of the comet and the tail length.

The presence of mussel hydrolysate during irradiation of the lymphocyte cells was found to decrease the comet parameters in a dose-dependent manner which further confirmed the radioprotective activity of mussel hydrolysate. These results could be comparable with the other commercially available known compounds such as gallic acid, ascorbic acid and diethyldithiocarbamate. Natural alkaloids isolated from a plant (*Vinca rosea*) popularly known as vinblastine sulfate has been reported to show significant

protection of the normal tissues against the γ-radiation-induced DNA damage on *in vivo* studies (Rajagopalan *et al*, 2003). Vinblastine sulfate has also inhibited the formation of radiation-induced DNA strand breaks in bone marrow cells (Rajagopalan *et al*, 2003).

The whole body exposure of mice to γ-radiation has increased the comet parameters of cells of various tissues and tumors because of damage to cellular DNA (Gandhi *et al*., 2003; 2004; Maurya *et al*., 2004). On the contrary, after intra-peritonial administration of glycyrrhizic acid in mice, a significant decrease in the comet parameters in bone marrow and blood cells has been demonstrated by Gandhi *et al*. (2004). These studies are further conformity of our results showing mussel hydrolysate as a potential compound for developing it as a drug for the protection of the normal cells from radiation-induced damages.

The ionizing radiation exposure to human has been increasing day by day that included medical X-ray, CT scans, nuclear accidents and also nuclear terrorism (Wilson, 2000). Under these serious deleterious nuclear hazards, a non-toxic compound from natural source such as mussel hydrolysate could be used as an ideal radioprotector. These compounds will also be useful in preventing diseases like cancer and degenerative diseases arising from gene aberrations. The mussel hydrolysate seems to inhibit the induction of single–strand damage in DNA by γ-radiation rather than protection of DNA damages itself. Further studies will be required to find out the actual mechanism of action of a purified compound from the mussel hydrolysate of Indian green mussel to develop as a potential radioprotecting drug useful in cancer therapy.

Fig 5.1: Effect of increasing doses of gamma radiation on pBR322 in presence and absence of mussel hydrolysate (25 mg/ml). a: Agarose gel electrophoresis of pBR322 exposed to increasing doses of gamma radiation.
Lane 1 represents unirradiated control without mussel hydrolysate while Lane 2 the same in presence of mussel hydrolysate. Lanes 3,5,7,9,11 represent plasmid DNA exposed to 30,60, 120,240,360 Gy in absence of mussel hydrolysate and Lanes 4,6,8,10,12 shows plasmid DNA exposed to 30,60, 120,240,360 Gy in presence of mussel hydrolysate

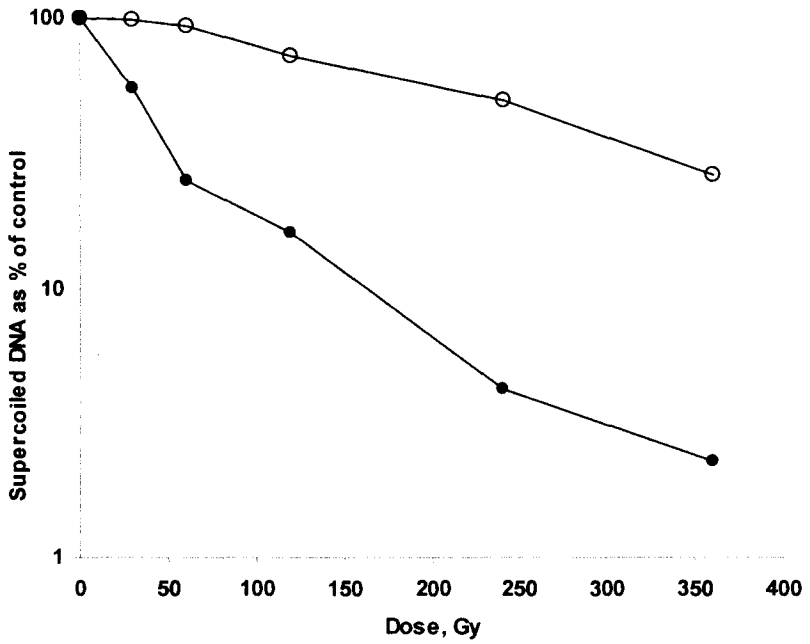


Fig 5.2: Graphical representation of data from Fig 5.1, ccc form expressed as % of control plotted against increasing doses of γ–radiation. Closed circle represent values of plasmid DNA in presence of mussel hydrolysate while open circles denote plasmid DNA without mussel hydrolysate

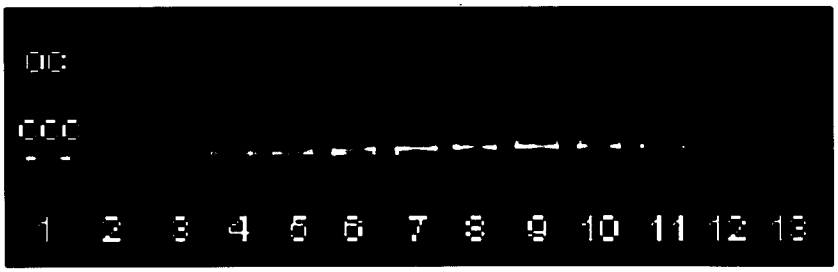


Fig 5.3: Effect of increasing concentration of mussel hydrolysate on γ-radiation induced damage to plasmid DNA (pBR 322). a: Agarose gel electrophoresis pBR322 exposed to 120 Gy of γ-radiation in the presence of increasing concentration of mussel hydrolysate. Lane 1 unirradiated plasmid + 50 mg/ml mussel hydrolysate , Lane 2 plasmid + radiation, Lane 3 to 11 and 13, plasmid + radiation + mussel hydrolysate (5, 15, 20, 25, 30, 35, 40, 45, 50 and 10 mg/ml respectively), Lane 12 unirradiated plasmid. The upper bands depict the open circular (oc) form while lower ones depict the supercoiled form (ccc).

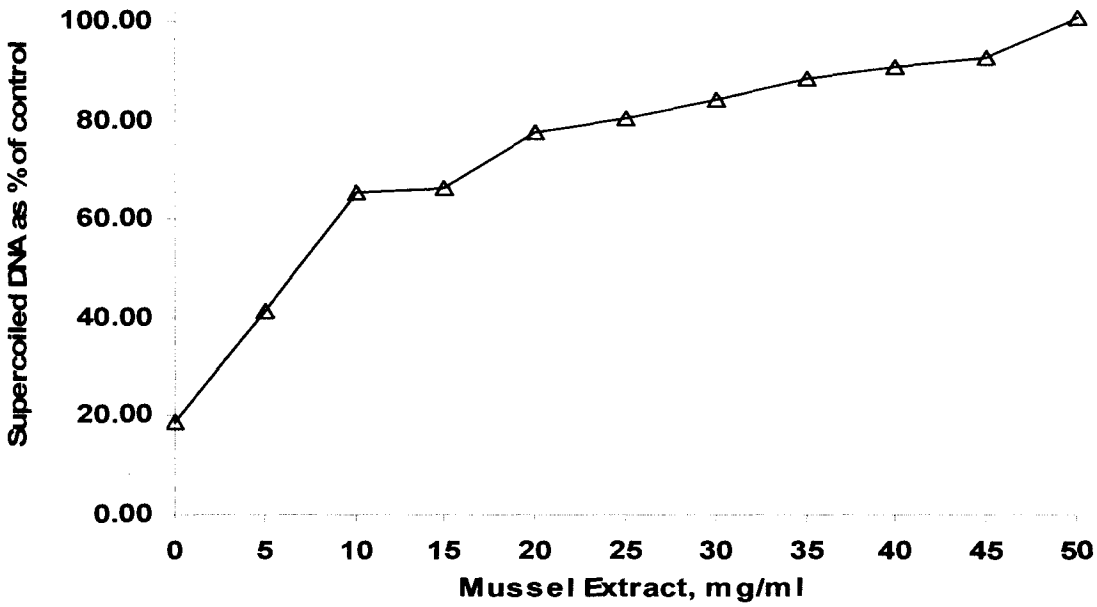
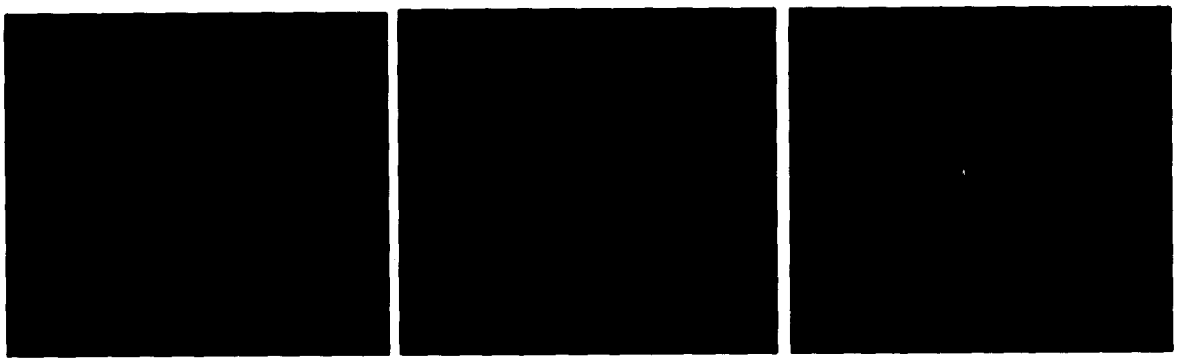


Fig 5.4: Graphical representation of data from Fig 5.2 a. ccc form expressed as percent of control plotted against increasing concentration of mussel hydrolysate.



A: Control B: 2 Gy C: Extract+2 Gy

Fig 5.5: Photomicrographs of isolated lymphocyte cells after Comet assay.
A: Control cells, B: Cells exposed to 2 Gy gamma radiation,
C: Cells treated with 0.5 mg/ml crude mussel hydrolysate and gamma radiation (2 Gy).

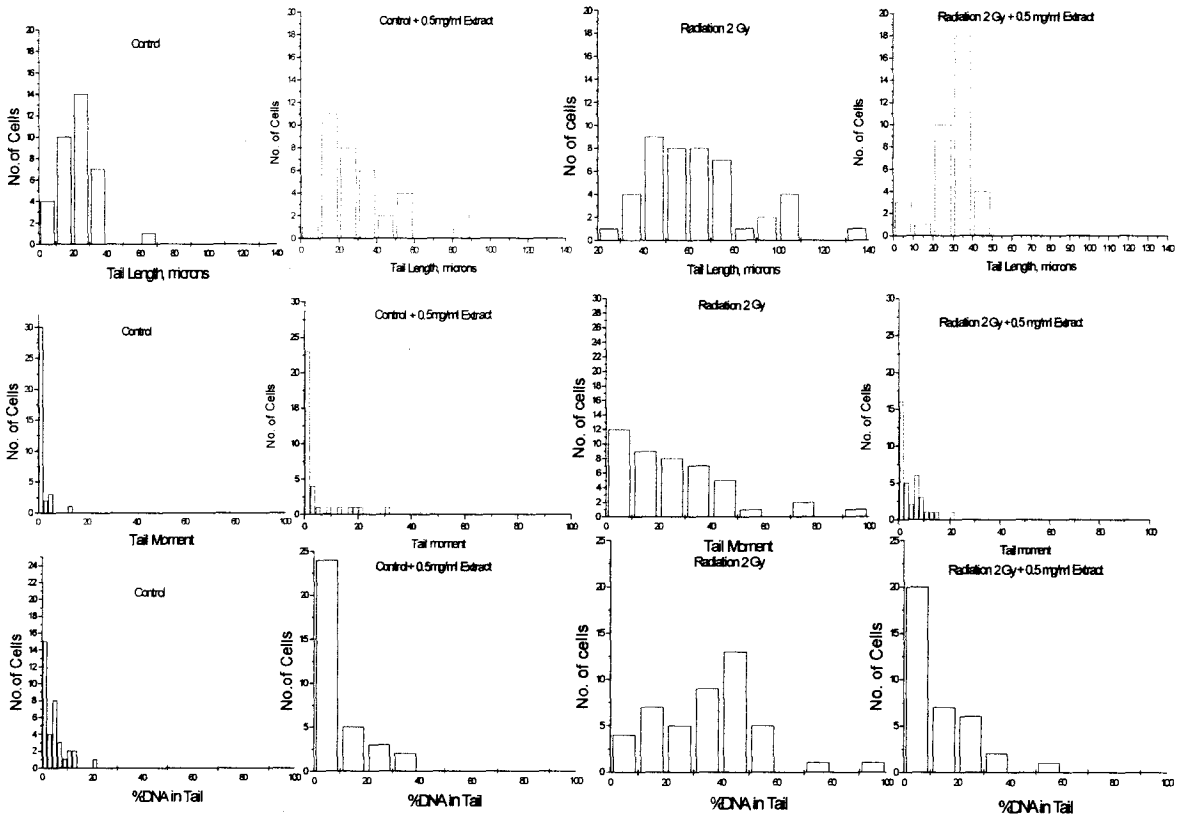
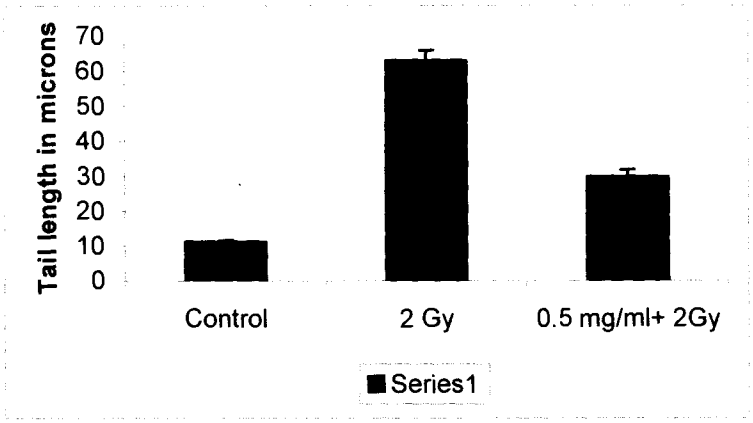
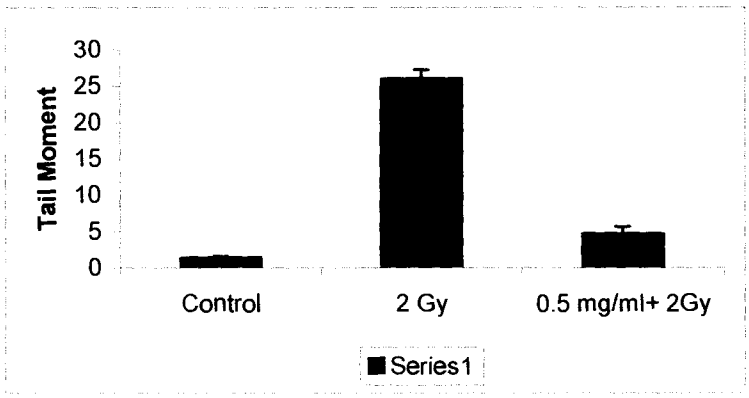


Fig 5.6: Frequency distribution pattern of Tail Length, Tail Moment and % DNA in Tail of isolated lymphocyte cells after assay. A: control cells, B: Cells treated with 0.5-mg/ml crude mussel hydrolysate. C: Cells exposed to gamma irradiation (2 Gy), D: Cells treated with 0.5-mg/ml crude mussel hydrolysate and gamma irradiation (2Gy).

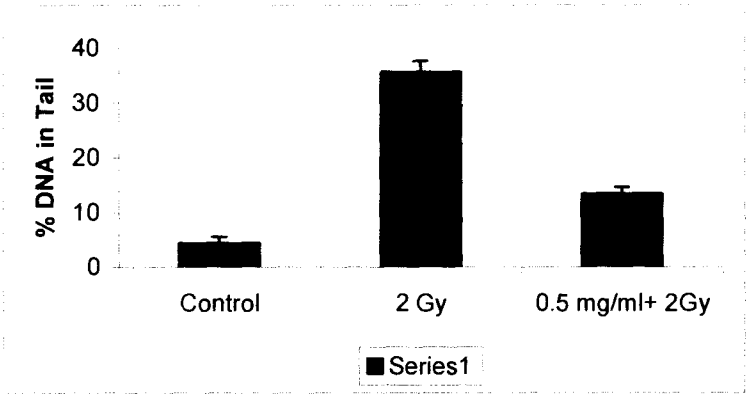


(A)

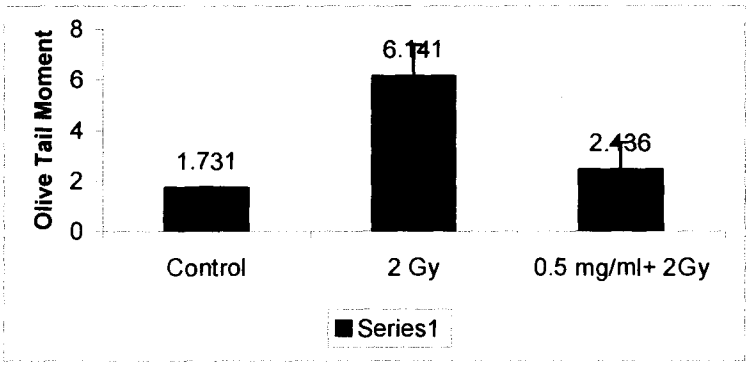


(B)

Fig 5.7 a: Reduction by mussel hydrolysate of γ–radiation induced nuclear DNA damage in lymphocytes as analyzed by Comet Assay. Mean (± S.D) of (A): Tail Length (TL); (B): Tail Moment (TM).



(C)



(D)

Fig 5.7 b: Reduction by mussel hydrolysate of γ–radiation induced nuclear DNA damage in lymphocytes as analyzed by Comet Assay. [Mean (± S.D) of (C): % DNA in Tail; (D) Olive Tail Moment].

CHAPTER 6

Evaluation of antioxidant enzymatic activity of mussel hydrolysate in the mice lymphocytes

Reactive Oxygen Species (ROS) are formed and degraded by all aerobic organisms, leading to physiological concentrations required for normal cell function or in excessive quantities, the state called oxidative stress. ROS are produced continuously in cells either as by-products of metabolism or deliberately as in phagocytosis (Cheeseman and Slater, 1993). There is a continuous generation of reactive oxygen species (ROS) in aerobic cells during normal metabolism. The role of mitochondria for generation of reactive oxygen species and electrons leak out to generate superoxide radical has been reported by Boveris and Chance (1973). It has also been estimated that 2-5% of all the electrons that travel down the respiratory chain, never make it to complex IV, but instead leak out from the respiratory chain to form $O_2^{\cdot -}$ and dismutation product H_2O_2 (Boveris, 1972). Other sources of reactive oxygen species include reactions of lipoxygenases, cyclooxygenases, xanthine oxidase and NADPH oxidase in phagocytic cells, D- amino acid oxidase and xanthine oxidase (Cross and Jones, 1991). Some of those molecules are extremely reactive, such as the hydroxyl radical while some are less reactive (superoxide and hydrogen peroxide).

Cellular membrane is one of the major targets of radiation damage as well as for oxidative attack. Damage to cell membrane and/or intracellular membranes would be expressed as an altered permeability resulting in transfer of unwanted molecules from one cellular compartment to another. This would produce unbalanced metabolism and finally lead to cell death. This is due to the lipid peroxidation of polyunsaturated fatty acid (PUFA) having double bonds that largely present in the phospholipid of membranes. Oxidative damage to lipids results in lipid peroxidation, which is the autoxidation of the polyunsaturated fatty acid side chains of lipids by a radical chain reaction. Unchecked per-oxidation of membrane lipid is catastrophic for living system. Lipid peroxidation results in the formation of malondialdehyde and 4-hydoxy-nonenal, which are mutagenic and can, react with DNA and proteins causing structural and/or functional damages to these macromolecules. To counter these deleterious effects of free radicals, cells acquire endogenous antioxidant defence system and scavenge ROS at different levels (Heng *et al.,* 2000). These include enzymatic and non-enzymatic substances. Antioxidant enzymes include Superoxide Dismutase (SOD), Catalase and

Glutathione peroxides (GPX). These substances scavenge the free radicals before they cause damage to cell vital components. Hence a balance between the pro-oxidant and antioxidant states in the cells is existed. However, when ROS overcome the defence systems of the cell and redox homeostasis is being altered, it resulted in oxidative stress. Oxidative stress is implicated in the pathogenesis of several disease including AIDS, Huntington's, Parkinson's disease, amyotropic lateral sclerosis, Alzheimer's disease and retinal degenerative disorders.

Antioxidant enzymes

An antioxidant is a compound, which reacted with free radicals and formed harmless un-reactive molecules protecting other biological molecules from damage. Antioxidants are either reactive chemicals such as vitamin E or specialised enzymes. An important part of the intracellular antioxidant defense system is antioxidant enzymes such as superoxide dismutase (SOD), catalase and peroxidases.

Superoxide Dismutase (SOD)

The enzyme superoxide dismutase (SOD, EC 1.15.1.1), catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide (Frei, 1994). As such, it is an important antioxidant that provided the defense to nearly all cells exposed to oxygen. Several common forms of SOD are proteins cofactored with copper and zinc or manganese or iron. The cytosols of virtually all eukaryotic cells contain SOD enzyme with copper and zinc (Cu-Zn-SOD). The Cu-Zn enzyme is a homodimer of molecular weight of 32,500. The two subunits are joined primarily by hydrophobic and electrostatic interactions. *E. coli* and many other bacteria also contain a form of enzyme with iron (Fe-SOD) (Yost and Fridovich, 1973). Some bacteria contain Fe-SOD, others Mn-SOD whereas some contain both. In humans, three forms of superoxide dismutase have been reported. Two copper/zinc containing SODs of which SOD1 is located in the cytoplasm whereas SOD3 is bound to the vascular endothelium (also called extracellular SOD) and the other one is manganese containing SOD2 which is located in the mitochondrial matrix (Frei, 1994).

The enzyme SOD dismutates two molecules of $O_2^{\cdot-}$ per reaction cycle which oxidises one molecule of $O_2^{\cdot-}$ to O_2 and with an electron release during this oxidation process. This finally reduces a second $O_2^{\cdot-}$ molecule to H_2O_2.

$$2\ O_2^{\cdot-} + 2H \rightarrow O_2 + H_2O_2 \qquad \text{(Frei, 1994)}$$

Catalase

Catalase (Ec 1.11.1.6) is an enzyme present in the cells of plants, animals and aerobic bacteria. It promotes the conversion of hydrogen peroxide, a powerful and potentially harmful oxidizing agent, to water and molecular oxygen (Diplock, 1994). Catalase is considered as one of the important antioxidant enzymes because it regulates H_2O_2 levels. So catalase neutralises the hydrogen peroxide toxicity that has been produced by all aerobic organisms ranging from bacteria to man. In addition to this, catalytic activity can also act in peroxidatic reactions involving substrates that readily donate hydrogen ions.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Catalase is located in a cell organelle called the peroxisome and also in the cytosol. Peroxisomes, in animal cells are involved in the oxidation of fatty acids, synthesis of cholesterol and bile acids (Frei, 1994). Each molecule of catalase is a tetramer of four polypeptide chains and each chain is composed of more than 500 amino acids. There are four porphyrin heme groups located within this tetramer and they are very much like the familiar hemoglobins, cytochromes, chlorophylls and nitrogen-fixing enzymes in legumes (Fita and Rossmann,1985). The heme group is responsible for catalase's enzymatic activity. Catalase has one of the highest turnover rates for all enzymes. One molecule of catalase can convert 8 million molecules of hydrogen peroxide to water and oxygen each minute (Michiels *et al.*, 1994).

Glutathion peroxidase (GSHPx)

Glutathione peroxidase is a selenoenzyme found in cytoplasmic and mitochondrial fractions. A deficiency of selenium dramatically decreases its activity (Michiels *et al.*, 1994). Like catalase, GSHPx hydrolyzes the H_2O_2 at low concentration (Grisham *et al.*, 1992). It primarily acts on lipid hydroperoxide (LHP) substrates which releases from the membrane phospholipids by phospholipase A2 (Van kuijk *et al.*, 1987).

It has been reported to utilise cholesterol hydroperoxide substrates as well (Thomas *et al.,* 1990). Other GSHPxs include a selenoglyco protein termed as plasma GSHPx (Takahashi *et al.,* 1987) where one insoluble enzyme associated with membranes called phospholipid hydroperoxide GSHPx (Ursini *et al.,* 1986). Besides the selenocysteine active site, GSH serves as an electron donor cofactor along with riboflavin, which maintains intracellular GSH levels (Thurnham, 1990).

Glutathione –S-transferase (GST)

Glutathione–S-transferase is a selenium independent enzyme having peroxidase activity (Michiels *et al.,* 1994). It is a multifunctional cytosolic, microsomal and membrane-bound phase II detoxifying enzyme (Mantle, 1995). It serves both in detoxification of exogenous electrophile and alkylating agents such as carcinogens as well as 4-hydroxy-2-3-nonenal - a cytotoxic byproduct of LHP metabolism (Leonarduzzi *et al.,* 1995; Singhal *et al.,* 1995). Owing to the peroxidase activity of LHP and phosphoilpid hydroperoxides, GST can be considered as a back up enzyme to CAT and GSHPx (Tsuchida *et al.,* 1992).

GSSG-R

GSSG-R is a flavoprotein that catalyzes the NADPH-dependent reduction of glutathione disulfide (GSSG). This reaction is essential for the availability of GSH levels *in vivo.* GSSG-R therefore, plays a major role in GSHPX and GST reactions as an adjuvant for the control of peroxides and radicals. When catalase levels are decreased, the GSH dependent enzymes become activated (Gastani *et al.,* 1994). In selenium deficiency, the GSHPX activity decreases but GSSG-R activity remains same (Castano *et al.,* 1995).

The antioxidant enzyme activity can be determined by direct and indirect ways. The difficulty in assaying SOD activity arises from the nature of its substrate $O_2^{\cdot-}$ which is a free radical with a short half-life in neutral aqueous solutions. More than 20 direct and indirect methods are described for assaying SOD activity (Singhal *et al.*, 1995). Spectroscopic and electroanalytical methods are currently used for superoxide measurements in biological samples.

Direct methods

In the direct method for determining the SOD like activity, $O_2^{\cdot-}$ is generated at initially relatively high concentrations (>1 μM). The decay of $O_2^{\cdot-}$ absorbance is followed spectrophotometrically in the UV region in the absence and presence of a testing compounds. This method affords the most reliable way of ascertaining SOD activity and its reaction mechanism. With this method, it is easy to descriminate between a catalytic and a non-catalytic compound. Under catalytic conditions, the initial concentration of $O_2^{\cdot-}$ is always exceeded in the tested compounds.

Spectroscopic methods include chemiluminescence and electron spin resonance spectroscopy. Most frequently used chemiluminescence method is based on measurement of the intensity of the fluorescence emitted after chemical oxidation of superoxide by lucigenin (Bis-N-methylacridium nitrate). Electron spin resonance also known as electron paramagnetic resonance and can be used to monitor molecules with unpaired electron including radicals such as $O_2^{\cdot-}$ (Castano *et al.*, 1995).

Indirect method

In indirect method, $O_2^{\cdot-}$ is generated either chemically or enzymatically with a constant flux and it is allowed to react with a detector molecule - D which scavenged the radical.

$$O_2^{\cdot-} + D \rightarrow O_2 + D^{\cdot}$$

The yield of D or the initial rate of its formation can be followed by its absorbance, luminescence, electron paramagnetic resonance etc.

Superoxide dismutase enzyme assay

Various authors have assayed superoxide dismutase activity by spectrophotometric method. Misra and Fridovich (1972) have assayed superoxide anion activity in terms of its ability to inhibit the radical-mediated chain-propagating autoxidation of epinephrine. Marklund and Marklund (1974) have assayed SOD by autoxidation of pyrogallol. Sun and Zigman (1978) assayed superoxide dismutase by autoxidation of epinephrine based on adrenochrome absorption at 480 nm. This method has further been improved to measure the absorption change at 320 nm. Misra and Fridovich (1972) have assayed superoxide anion by autoxidation of epinephrine.

Catalase enzyme assay

Aebi *et. al.* (1974) have assayed catalase enzyme activity spectrophotometrically.The decomposition of hydrogen peroxide (H_2O_2) catalysed can be recorded by UV spectrum due to the absorbance of H_2O_2 in this region. At 240 nm, the molar extinction coefficient for H_2O_2 is 43.6 mol^{-1}.

In the present chapter the effect of mussel hydrolysate on antioxidant enzymes superoxide dismutase and catalase in isolated mice lymphocyte cells was studied and reported.

MATERIALS AND METHOD

Chemicals: Fetal Calf Serum (FCS), RPMI-1640, Trypan blue, Ammonium Chloride, Follin's Reagent, Epinephrine, Hydrogen peroxide, Sodium Chloride, EDTA, Tris HCl, Triton X-100 and Catalase enzyme. All chemical used were of highly purified grade.

Mussel hydrolysate of concentrations 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml were used to see the effect on antioxidant enzymes in mice lymphocyte cells. Required concentrations of all these test samples were prepared in terms of weight by volume ratio in RPMI-1640 medium.

Preparation of Reagents

A: <u>For Isolation of Lymphocyte:</u>

❖ <u>Phosphate Buffer Saline (10 mM, pH =7.4)</u>

Composition: Na_2HPO_4 - 10 mM

NaH_2PO_4 - 10 mM

Saline (NaCl) - 0.84%

Preparation: 10 mM NaH_2PO_4, 10 mM Na_2HPO_4 and 0.84% saline were added to 800 ml of distilled water and pH was adjusted to 7.4. The final volume was made to 1000 ml.

❖ <u>RPMI-1640 Media (for 1 liter):</u>

Composition: $NaHCO_3$ – 3.7 g

Penicillin – 60 mg

Streptomycin – 250 mg

HEPES – 5.67 g

L-glutamine – 2.92 g

Preparation: 3.7 g $NaHCO_3$, 5.67 g HEPES, 2.92 g L-glutamine, 60 mg Penicillin, 250 mg Streptomycin (in the same serial order) were added to the 700 ml of autoclaved distilled water. The RPMI-1640 powdered was added with constant stirring to avoid clump formation, pH was adjusted to 7.4 and the volume was made to 1000 ml with

autoclaved distilled water. The media was filtered and stored in a bottle at 4° C. The sterile condition was always maintained for preparing media to avoid contamination.

B: For RBC lysing buffer:

0.83% NH_4Cl was used for lysis of RBC cells.

C: For spleen cell counting stain

0.4% Trypan blue was used for counting spleen cells.

D: For Lysis buffer

Composition: 20 mM Tris-Hcl pH 7.4
2 mM EDTA
2 mM EGTA
0.1 mM Phenyl Methyl Sulphonyl Fluoride (PMSF)
1% Triton X 100
Protease inhibitor

(E) <u>For the Estimation of Protein by Lowry-Follin's Method:</u>

- <u>Reagent A</u>: 1% of Sodium Potassium Tatarate
  2% of Sodium Carbonate in 0.1N NaOH.
- <u>Reagent B:</u> 1% $CuSO_4$.
  For making Lowry's Reagent, 50.0 ml of reagent A &1.0 ml of reagent B were used.
- Follin's Reagent (50%): Make 1:2 dilutions with distilled water.

(F) <u>For Superoxide Dismutase (SOD) Assay:</u>

a. <u>Sodium Carbonate Buffer (10x, pH =10.0):-</u>

Composition: Sodium carbonate – 50 mM
Sodium bicarbonate – 50 mM
EDTA – 50 mM

Preparation: In 80 ml of distilled water, 50 mM sodium carbonate, 50 mM sodium bicarbonate, 50 mM EDTA were added and then pH was adjusted to 10.0. The final volume was made to 100 ml with distilled water. Before using the buffer was diluted to a strength of 1X.

b. Epinephrine – 20 mM.

(G) For Catalase Assay:-

a. Phosphate Buffer (10x, pH = 6.8):-

Composition: Dipotassium Hydrogen Peroxide – 50 mM

Dihydrogen potassium Phosphate – 50 mM

Preparation: In 30 ml of distilled water 50 mM Dipotassium Hydrogen Peroxide, 50 mM Dihydrogen Potassium Phosphate were added. After adjusting the pH to 6.8, the volume was made to 50 ml with distilled water. Before using the buffer was diluted to 1X.

b. Phosphate buffered Hydrogen Peroxide– 17 mM

Animal maintenance

Eight to ten week old BALB/c male mice weighing approximately 20-25 g were selected from inbred group maintained under standard conditions at temperature of 25± 2° C and humidity with 12-hour light and 12-hour dark cycles. The animals were fed with commercially available libitum feed. Usually four animals were housed in each sterile polypropylene cage containing a sterile paddy as bedding. All animal experiments were conducted with strict adherence to ethical guidelines laid down by the committee for the purpose of control and supervision of experiments on animals (CPCSEA) constituted by the Animal Welfare Division of the Government of India for the use of animals in scientific research.

Isolation of mice lymphocyte cells

Animals were killed humanely by cervical dislocation. Spleen was removed from the animals and kept in cold RPMI-1640 media containing 15 mM HEPES, 2 mM L glutamine, 100 IU/ml pencillin, 100 mg/ml streptomycin and 20 μM 2-mercaptoethanol. Spleen was then gently crushed with the help of the crusher and centrifuged at 2000 rpm for 3 minutes. The supernatant was discarded and 3 ml of cold 0.83% NH_4Cl was added to the pellet and kept exactly for 3 minutes with proper mixing for the lysis of the RBCs cells. Cell suspension was diluted with the RPMI-1640 media and centrifuged at 2000 rpm for 3 minutes. The supernatant was again discarded. The pellets were resuspended

in cold RPMI-1640 media and centrifuged at 2000 rpm for 3 minutes for two times. The supernatant were discarded and the pellets were resuspended in 5 ml of RPMI-1640 media and lymphocytes were isolated for further use.

Experimental design

Mice lymphocytes, $2x10^6$ cells/ml were cultured in RPMI-1640 media with 5% FCS and incubated for 24 hours with mussel hydrolysate of concentrations of 0.25 mg/ml, 0.5 mg/ml and 1.0 mg/ml. After incubation cells were lysed by means of lysis buffer and protein estimation was done by Lowry Folin's method. Superoxide dismutase and catalase assay were done according to the following protocol:

(A) Method for estimating Superoxide Dismutase (SOD)

Principle: **Epinephrine auto-oxidation inhibition method**

Superoxide dismutase enzyme assay was done according to Sun and Zigman (1978) where auto-oxidation of epinephrine was recorded at pH 10.0. It was converted to an adrenochrome (a pink colored solution) absorbing itself at 320 nm. SOD in the sample also scavenged the O^{2-} radicals and converted into H_2O_2 which prevented the conversion of Epinephrine to Adrenochrome.

$$\text{Epinephrine} \xrightarrow{O^{2-}} \text{Adenochrome}$$

Protocol: (i) *For standard (only for Epinephrine)*

Sample	Test	Blank
SOD buffer	955 µl	980 µl
	Auto zero	
Epinephrine	25 µl	-
	The absorbance was recorded at 320 nm for 3 min	

(ii) *For Cell Lysate*

Sample	Test	Blank
SOD buffer	965 µl	990 µl
Cell lysate	10 µl	10 µl
	Autozero	
Epinephrine	25 µl	-
	The absorbance was recorded at 320 nm for 3 min	

Calculation: Equation used for the calculation of specific activity of superoxide dismutase enzyme was as follows:

Specific activity = (ΔA/min x 30) / P

where, A denotes change in absorbance of epinephrine due to the activity of the enzyme present in the sample and P the amount of protein (mg) per ml of reaction mixture present in the sample.

(B) Method for estimating Catalase (CAT):-

Principle: **Spectrophotometric analysis of catalase**

The decomposition of hydrogen peroxide (H_2O_2) catalyzed by catalase is recorded by UV spectrum due to the absorbance of H_2O_2 in this region. At 240 nm, the molar extinction coefficient for H_2O_2 was 43.6 mol^{-1} (Chance and Malhey, 1955).

Catalase standard curve was made by plotting enzyme unit/ml on X-axis and change in absorbance of H_2O_2 on Y-axis.

Protocol:

(i) 1 ml of phosphate buffer in both test and blank cuvette was taken and, autozero was adjusted.

(ii) The test cuvette was removed and 1 ml Phosphate Buffered H_2O_2 was added. The absorbance was recorded at 240 nm for 3 mins.

(iii) *For Cell Lysate*:

Sample	Test	Blank
Phosphate buffer	-	990 µl
Phosphate buffered H_2O_2	990 µl	-
Sample	10 µl	10 µl
	The absorbance was recorded at 240 nm for both T_0 and T_3 by time scanning for 3 min in the spectra manager	

Calculations:

Specific activity of the enzyme at a temperature of 25°C defined in terms of micromoles of H_2O_2 consumed per minute per milligram of protein sample and expressed as unit of enzyme as follows:

$$\textbf{Specific activity} = \frac{\Delta A/\text{min} \times 1000}{43.6 \times P} \textbf{ units per mg protein (U/mg protein)}$$

Where, ΔA denotes change in absorbance of Hydrogen peroxide due to the activity of the enzyme present in the sample, P the amount of protein present (mg) per ml of reaction mixture.

Statistical Analysis

The statistical analysis for the above assays was done using students *t* – test.

RESULTS

The catalase enzyme activity was assessed using mussel hydrolysate on mice lymphocyte cells. A straight line relationship (y=0.0078 x, r^2= 0.9694) was obtained when catalase standard curve plotted by taking catalase enzyme units on X-axis and change in absorbance of hydrogen peroxide on Y-axis (Fig. 6.1).

Mussel hydrolysate concentration of 0.5 mg/ml significantly ($p<0.05$) enhanced the activity of catalase enzyme. A higher value of 13.222 catalase units/mg protein was recorded as compared to control experiments where the value was 9.6145 catalase units/mg protein (Table 6.1). A concentration of 1.0 mg/ml was found to reduce the enzyme activity, slightly where a value of 7.096 catalase units/mg protein was observed (Table 6.1 and Fig. 6.2).

These results showed that a 37% more activity than the control was recorded when the lymphocyte cells were incubated for 24 hours with 0.5 mg/ml of mussel hydrolysate. However, there was a significant reduction of 26% in the activity of enzyme when the lymphocyte cells were incubated with 1.0 mg/ml of mussel hydrolysate.

Similarly, the SOD activity was found to enhance when the mice lymphocyte cells were treated with mussel hydrolysate concentrations of 0.25 mg/ml and 0.5 mg/ml (Table 6.2 and Fig. 6.3). A value of 16.085 SOD units/mg protein was recorded at 0.25 mg/ml concentration of mussel hydrolysate. A significant increase ($p<0.001$) of 20.087 SOD units/mg proteins was recorded at 0.5 mg/ml concentration of mussel hydrolysate. This indicated that there was a slight increase in the SOD activity (2.6%) at 0.25 mg/ml concentration whereas at 0.5 mg/ml the enzyme activity was significantly increased by 28% (Table 6.2).

DISCUSSION

In normal healthy cells, enzymatic and non-enzymatic antioxidants serve to balance the intracellular concentration of ROS, thereby delaying or inhibiting the destructive oxidation of molecular components within the cellular mileu by ROS (Singhal *et al.*, 1995). The potential for oxidative damage is greatly augmented if the antioxidant buffering capacity is insufficiently expressed (Haddad *et al.*, 2000). Many studies suggested that antioxidant systems are critical in protecting against tumor promoting agents and one or more components of these systems are deficient in many forms of cancer. Interestingly, cell malignancy or transformation is often accompanied by a decrease in activity of antioxidant enzyme (SOD, catalase, GSH-Px, GR) that has been reported to increase the cell sensitivity to pro-oxidant compounds (Sergediene *et al.*, 1999; Brevard *et al.*, 2002).

In the present study mussel hydrolysate of 0.5 mg/ml concentration enhanced the activity of both catalase and superoxide dismutase enzyme after its incubation for 24 hrs with lymphocyte cells. There was 37% more activity of catalase than the control when the lymphocyte cells were treated with 0.5 mg/ml of mussel hydrolysate. This enhancement of enzyme activity has further been confirmed the radioprotective activity of mussel hydrolysate. There was a slight decrease in the catalase activity when the lymphocyte cells were treated with 1.0 mg/ml of mussel hydrolysate. This may be due to the pro-oxidant activity of mussel hydrolysate at higher concentrations. A gradual enhancement of SOD activity was observed when the mice lymphocyte cells were treated with mussel hydrolysate at concentrations of 0.25 mg/ml and 0.5 mg/ml. There was about 28% enhancement of SOD activity at concentration of 0.5 mg/ml of mussel hydrolysate. There was also an enhancement of SOD activity at 0.25 mg/ml concentration of mussel hydrolysate, which was negligible.

The free radical scavenging activity of mussel hydrolysate has been discussed in previous Chapter-4 where an increase in the activity from 48% at a concentration of 0.1 mg/ml to 80% at a concentration of 10.0 mg/ml of mussel hydrolysate was reported. This shows that the mussel hydrolysate possess free radical scavenging activity. The

present study showed that it also enhanced the antioxidant enzyme activity. Both these characters are important for radioprotective activity and the present study confirms the presence of radioprotection activity in mussel hydrolysate.

Reactive oxygen species are highly reactive due to the presence of unpaired electron and readily react with most biomolecules initiating a chain reaction like reactions. ROS causes damage to DNA, lipids and proteins resulting in several biological effects ranging from alterations in signal transduction and gene expressions to mitogenesis and cell death. Ionizing radiations like gamma rays and X-rays are also cause damage to living cells/ organisms essentially due to direct and indirect actions (Altman, *et al.,* 1970). DNA is the principal cellular target of ionizing radiation. The nuclear region of cell is 100 times sensitive than cytoplasm. The damage to DNA includes single strand or double strand breakage which in turn leads to cell division delay, formation of modified cell, neoplastic formation, point mutation, chromosomal aberration, cell loss and ultimately the cell death. Ionizing radiation also affects other macromolecules like proteins and lipids. Enzyme may loss their active site due to changes in their 3-D structures.

Several natural compounds have both free radical scavenging and antioxidant activities. These compounds act as effective radioprotectors. Santhosh Kumar *et. al.* (2004) have found that chlorophyllin, a water-soluble mixture of sodium-copper salt of the green plant pigment chlorophyll, at concentration of 0.1 -10 μm enhanced the activity of catalase and glutathione peroxidase in mice lymphocyte cells after 24 hours where SOD activity remained same in control cells. After 72 hrs, both catalase and SOD activity has been reported to increase, significantly (Santhosh Kumar *et. al.,* 2004). Chlorophyllin also showed free radical scavenging activity at concentrations of 0.1 -10 μm. Sushma *et. al.* (2005) have studied the radiosensitive effect of ellagic acid on Hela cell line implanted in mice and reported that ellagic acid of 100 μmol/L concentration significantly decreased the activity of SOD, catalase and GPX. The susceptibility of tumor cells to radiation or drug is related to a decrease degree of antioxidant activity. These studies showed the relationship between antioxidant enzyme activity and radiation sensitivity.

Thus the present study showed that mussel hydrolysate at concentrations of 0.25 mg/ml to 0.5 mg/ml enhanced the activity of antioxidant enzymes catalase and superoxide dismutase in mice lymphocytes, significantly. Hence, this characteristic of mussel hydrolysate can be attributed to its radioprotective properties.

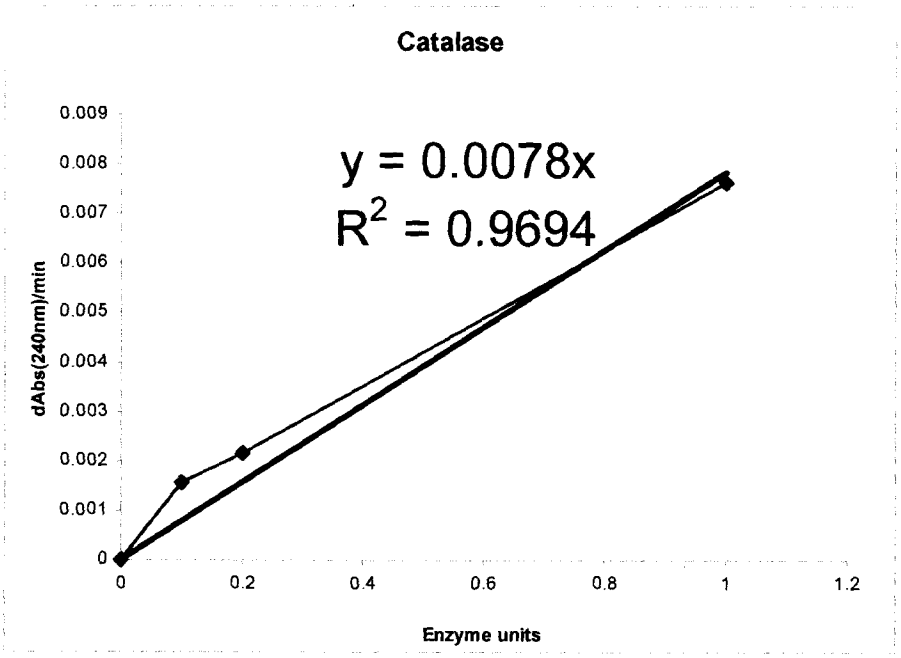


Fig. 6.1. Catalase standard curve plotted with absorbance

Table 6.1. Effect of mussel hydrolysate on catalase enzyme in isolated mice lymphocyte cells

Extract mg/ml	ΔAbs/min	Units of CAT	Protein (μg)	Catalase Units/mg protein	Mean	SD
Control	0.00484	0.621282	60.8036	10.218	9.6145	± 1.012
	0.00535	0.685897		8.296		
	0.00492	0.631791		9.374		
	0.00512	0.642732		10.57		
0.5 mg/ml	0.00714	0.915641	67.3958	13.586	13.22275	± 2.05556
	0.00537	0.688461	67.3958	10.215		
	0.00765	0.981666	67.3958	14.566		
	0.00756	0.978621	67.3958	14.524		
1.0 mg/ml	0.00772	0.92812	127.812	7.261	7.096	± 0.124478
	0.00691	0.89208	127.812	6.979		
	0.00732	0.91012	127.812	7.119		
	0.00701	0.89820	127.812	7.025		

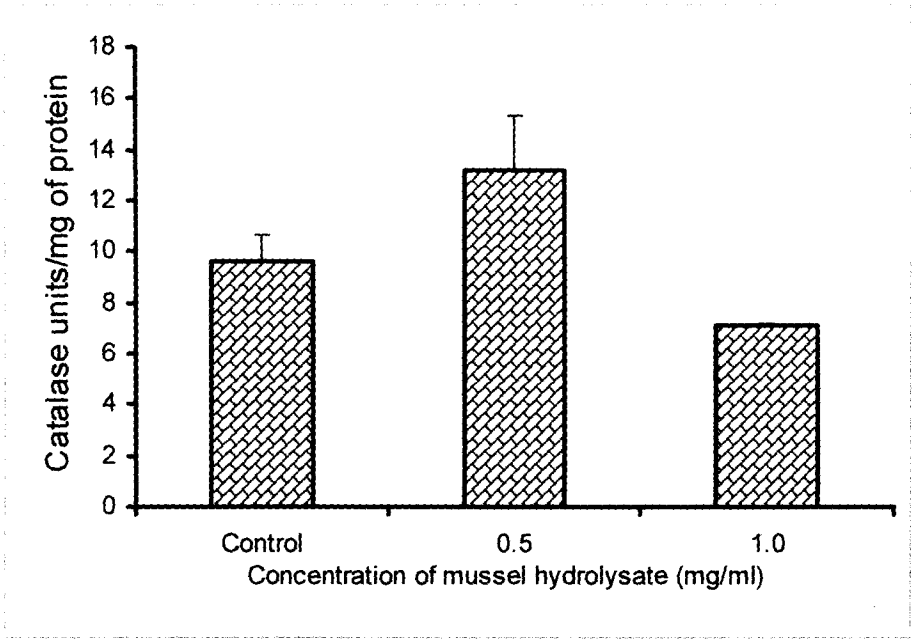


Fig. 6.2. Changes in antioxidant enzyme catalase in mice lymphocytes after 24 hrs of incubation with mussel hydrolysate at concentrations 0.5 and 1.0 mg/ml.

Table 6.2. Effect of mussel hydrolysate on SOD enzyme in isolated mice lymphocyte cells

Sample	0 sec	180sec	dAbs/ min	Epinephrine rate	% Inhibition	Units of SOD	Protein added (µg)	SOD Units/mg protein	Mean	SD
	a	b	c	d	e	f	g	h		
			b-a/3	Mean	(d-c /d)*100	e/50		f*1000/g		
Epinephrine auto-oxidation	0.3013	0.4437	0.04747	0.0466						
	0.2925	0.4340	0.04717							
	0.2954	0.4309	0.04518							
Control	0.3171	0.4306	0.03786	0.0466	18.77	0.375	24.32143	15.4373	15.6846	± 0.39
	0.3198	0.4326	0.03759	0.0466	19.35	0.387	24.32143	15.9136		
	0.3071	0.4209	0.03795	0.0466	18.58	0.372	24.32143	15.2785		
	0.3082	0.4206	0.03747	0.0466	19.59	0.392	24.32143	16.1093		
Cells + 0.25 mg/ml	0.3198	0.4294	0.0365	0.0466	21.61	0.432	25.2	17.15	16.085	± 0.72
	0.3071	0.4195	0.0375	0.0466	19.60	0.392	25.2	15.56		
	0.3068	0.4185	0.0372	0.0466	20.11	0.402	25.2	15.96		
	0.3070	0.4192	0.0374	0.0466	19.75	0.395	25.2	15.67		
Cells + 0.5 mg/ml	0.3047	0.4068	0.0340	0.0466	26.98	0.5396	33.06944	20.016	20.0787	± 0.16
	0.3075	0.4092	0.0338	0.0466	27.28	0.5456	33.06944	20.238		
	0.3231	0.4254	0.0341	0.0466	26.81	0.5362	33.06944	19.889		
	0.3046	0.4063	0.0339	0.0466	27.19	0.5438	33.06944	20.172		

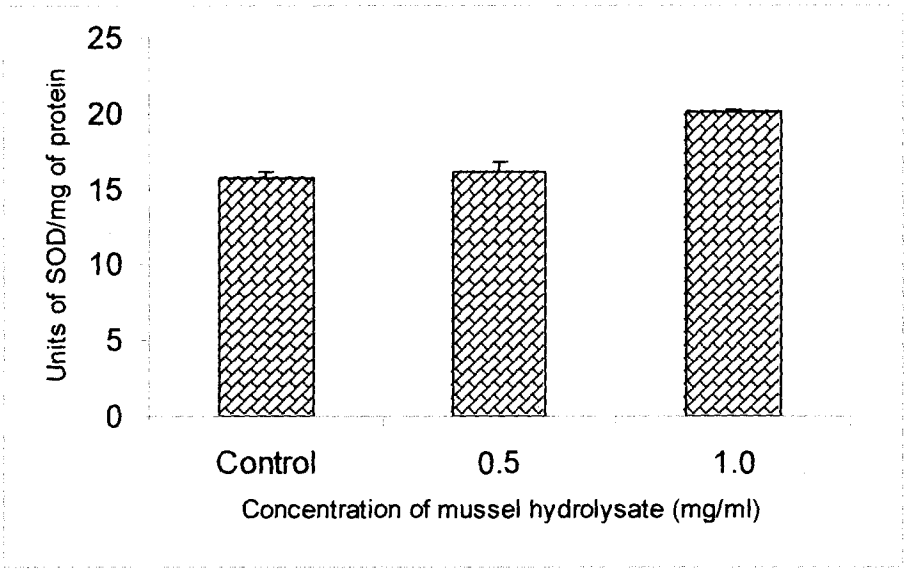


Fig. 6.3. Changes in antioxidant enzyme SOD in mice lymphocytes after 24 hrs of incubation with mussel hydrolysate at concentrations 0.25 and 0.5 mg/ml.

CHAPTER 7

Effect of different environmental parameters of the habitat of green mussels on the infectious activity of mussel hydrolysate

Knowledge of prevailing environmental factors is essential for understanding the ecology and interrelations of the organisms inhabiting the area. The occurrence of species, their distributional patterns, variations in plankton communities and assemblages have reported to be influenced by environmental factors significantly (Omori and Ikeda, 1984).

Temperature is probably the most important and best-studied environmental factor affecting marine organisms. It could act as limiting factor for the growth, reproduction and distribution of the organisms especially in temperate waters. Salinity next to temperature is the other important environmental factor affecting marine organisms. Salinity has been reported to affect the distribution and reproduction of animals (Rivonkar, 1991). From the physiological point of view, the significance of salinity lies in the osmotic pressure (Friedrich, 1969). Similarly, oxygen is the other parameter vital for all living beings and played a very important role with respect to marine life. Except for certain bacteria and a few animals (anaerobic organisms), oxygen is essential to all forms of marine life for their survival. Dissolved oxygen is an index to the health of an aquatic system and hence it is considered as a natural indicator to evaluate prevailing pollution load.

Goa has coastline of about 106 km (14^{0} 51 to 15^{0} 48 ! and 73^{0} 41 to 74^{0} 20 ! E) and is bounded by the Western Ghats in the east and by the Arabian Sea in the west. The coast trends in a NNW-SSE direction and consists of sandy beaches separated from one another by rocky headlands and river openings (Anon, 1978). All together, nine major and minor rivers flow through the hills and plains of Goa. However, the Mandovi and Zuari are two important rivers interspaced by Cumbarjua canal forming a major estuarine complex The Zuari river, originates in Dighi ghats of the Sahayadri mountain ranges (Western Ghat) in Karnataka and after a prolonged flow of 67 kms, empties into the Arabian Sea at Dona Paula. The average runoff of Zuari estuary is 9.0 km^{3} $annum^{-1}$

(NIO, Tech. Report, 1979). Dona Paula Bay is protected towards north, east and the south by rocky promontories. It is less than 2.0 kms upstream of the opening of Zuari River in the Arabian sea. A semi enclosed area, in the Dona Paula Bay, located at the mouth of the river Zuari (15 ^{0} 27 N lat. and 73 ^{0} 47 E long.) was chosen for monitoring the environmental parameters for the present study.

The tidal influence in the study area has been reported to be comparatively low (<2.0 m) in monsoon (June- September) (Dehadrai, 1970; Singbal, 1973). Similarly, the wave action is reported to be the minimum, thus facilitating protection and steady growth of culture animals round the year (Parulekar *et al.,* 1982). The bottom deposits in Dona Paula bay have been reported to be firmed (Parulekar and Dwevedi, 1974).

In the present study an attempt was made to see the effects of different environmental parameters such as temperature, salinity, dissolved oxygen, pH, particulate organic carbon, chlorophyll a and suspended load on the quality of mussel hydrolysate which was determined taking into consideration the infectious activity as one of the important indicators of biological activity. The infectious activity of mussel hydrolysate was assessed in terms of their anti-viral properties using standard haem-agglutination assay.

MATERIAL AND METHODS

In the present study, seawater samples were analysed for the environmental parameters such as temperature, salinity, dissolved oxygen, pH, particulate organic carbon, chlorophyll a and suspended load. The air and water temperatures were immediately measured using a portable glass thermometer (accuracy: 0.1° C).

Water samples for salinity were collected from the surface in plastic bottle. In the laboratory the salinity of the samples was measured using a portable refractometer (Atago). Salinity values were expressed as parts per thousand (ppt).

The dissolved oxygen was measured by the classical chemical method as commonly known as Winkler Iodometric titration method (Parsons *et al.*, 1984). Water samples (in triplicate) from surface zone were collected in 125 ml oxygen bottles. Immediately upon the collection, Winkler A and B reagents were added to fix the samples. The samples were then stored away from the sunlight in portable ice box. In the laboratory, the samples were titrated against standard sodium thiosulphate solution using starch as an indicator. The values were expressed as ml/liter of the seawater (Parsons *et al.*, 1984). The pH of the water was determined with the help of a portable pH meter (Philip: PP 9046).

For the analysis of particulate organic carbon (POC) and chlorophyll a, water samples (in triplicate) were collected in one-liter polythene containers. Immediately after collection, two aliquots of 1.0 to 2.0 liters were filtered through previously combusted GF/C glass fiber filter in muffle furnace (450° C for 3 hrs). Filter papers were then washed with distilled water to remove salt and then frozen at -20° C until further analysis. The particulate matter left on the filter paper was analyzed for POC and chlorophyll a.

Particulate Organic Carbon (POC) was analyzed following the method as described by Parsons *et al.* (1984) using sulphuric acid-dichromate reagent. This method involved wet oxidation of carbon by acid dichromate. Decrease in extinction of the yellow dichromate solution was taken as a measure of the carbon.

The Chlorophyll a was determined flurometrically on a Turner Design flurometer after overnight extraction of the filter papers in 90% acetone in dark bottles at a temperature of 5^0 C (Parson *et al.,* 1984). The values were expressed as µg/litre of seawater. Suspended solids in the water samples were analyzed by the standard method of Parsons *et al.* (1984).

Evaluation of antiviral activity

The antiviral activity of the mussel hydrolysate prepared from the Indian green mussel (*Perna viridis*) during different months from September' 02 to June' 03 was assessed at the Pasteur Institute of Epidemiology and Microbiology, St' Petersburg, Russia. Due to monsoon during July to September' 2003, samples of green mussels was not found. The following protocol was used for the assessment bioactivity of mussel hydrolysate in terms of anti-viral activity:

A- Preparation of Influenza virus titer

Stock solution of Influenza viral strain A (Mississippi/85/H_3N_2) was diluted in seven test tubes with peptonic medium in descending order to achieve virus concentrations from 10^{-2} to 10^{-7}.

B- Preparation of peptonic medium

Gelatin (2.0 g) was thoroughly mixed in 100 ml double distilled water. This was followed by addition of Nacl (8.0 g), KCl (0.6 g), $CaCl_2$ (0.8 g), $MgCl_2$ (0.15g), glucose (0.9 g) and 25 ml of phenol (0.01%). The volume of the solution was made up to 1000 ml with double distilled water. 0.1 g of antibiotic (Penicilline: 1000 units /ml) was also added and pH of the solution maintained between 6.5 and 7.0. The solution was sterilized in an autoclave for one hour at 15 lbs. The pH of the medium was adjusted with $NaHCO_3$ to achieve a pH of 6.0 before the experiment.

C- Preparation of extract

0.5 ml of mussel hydrolysate was transferred to one test tube (marked as N_1) containing 4.5 ml of peptonic solution to achieve dilution of extract 1/10 times.

D- Preparation of *in vitro* experiment with Vital Chorio-allantoic (VCA) membrane of chick embryo and viral strain A (Mississippi/85/H_3N_2)

One set of multiwell plate was thoroughly cleaned with soap and washed several times with double distilled water and then with alcohol for sterilization. Two fertilized eggs of chicken (13 days old) were cleaned with freshwater to remove all impurities. The eggs were also cleaned thoroughly with absolute alcohol. A small cut was made at the narrow end of the eggs with a help of a pair of sharp scissors and shell piece was removed. The egg with growing embryo was emptied in a glass bowl. Small pieces of the shell with vital chorio allantoic (5x5 mm) were cut and transferred immediately in the peptonic medium.

The multiwell was divided into three columns with four wells in each column and 6 wells in each row. In all well of the multiwell plates, 0.5 ml of peptonic medium was transferred with a sterilized pipette. A single piece of the shell with VCA cut from the fertilized egg was transferred in the well of multiwell plate so as to completely dip in the peptonic medium.

0.1 ml of mussel hydrolysate (1/10) was pipetted from N_1 test tube and transferred in all wells of column 1. The second column was identified as control preparation column where only mussel hydrolysate was transferred in first row of four wells. The multiwell plate was finally closed properly and kept at a constant temperature of $34\pm1^{\circ}$ C for 2 hours.

The multiwell plate was removed from oven after 2 hours and in each well of columns 1 & 3, 0.1 ml of influenza virus was added in the descending dilution i.e. from 1^{st} row (10^{-2}) to 6^{th} row (10^{-7}). The virus was not added in the wells of control preparation i.e. column-2. The plate was again closed properly and kept at a constant temperature of $36\pm1^{\circ}$ C for incubation for 48 hours.

The multiwell plate was removed from the oven after 48 hours and shell pieces taken out carefully with the help of fine tweezers avoiding the mixing of the solution of the wells. In each well of all the three columns 0.05 ml of chicken erythrocyte (5%) was added with the help of a pipette. The plate was kept undisturbed at room temperature for 20-30 minutes and then assessed for antiviral activity by hem-agglutination reaction.

RESULTS

The variation in air temperature from September' 02 to June' 03 was between 29.0 and 34.5° C. Minimum air temperature was recorded in the month of September' 02 whereas maximum during November' 02 and June' 03 (Fig. 7.1). The variation in air temperature throughout the study period was 5.5° C (Fig. 7.1). Similarly the temperature of water column fluctuated between 25.8 and 31.6° C with a variation of 5.8° C (Fig. 7.2). Minimum value of water temperature (25.8° C) was recorded during January' 03 whereas maximum during May and June' 03 (31.6° C). The values of infectious activity from September' 02 to June' 03 were plotted Figs 7.1 & 7.2 along with temperatures to see the effect of these parameters on the activity. No significant difference was observed between air and water temperatures of the environment and the infectious activity of the mussel hydrolysate during the study period. This clearly indicated that the influence of temperature on the quality of mussel hydrolysate was negligible.

The pH of the water column was varied form 7.1 to 8.0. Minimum values of pH were recorded during May' 03 whereas the maximum in October' 02 (Fig. 7. 3). The values of infectious activity were plotted against the pH where no significant correlation was observed ($p>0.005$).

A marked difference of 10 ppt in the values of salinity was recorded during different months of the study period. The minimum salinity value (25 ppt) was obtained in September' 02 whereas, maximum in May' 03 (35 ppt) (Fig. 7.4). The salinity values showed that the water was less saline during July to September, which coincided with the monsoon seasons. Monthly variations in infectious activity and salinity did not show any significant correlation.

The variation in dissolved oxygen value was less (1.4 ml/l) indicating the most stable conditions of the environment. Minimum values (3.2 ml/l) were recorded during December' 02 and the maximum (4.6 ppt) during September' 02 (Fig. 7.5). No significant correlation was observed between infectious activity and dissolved oxygen.

The variation in particulate organic carbon during different months is depicted in Figure 7.6. The minimum values (0.50 µg/l) were observed in February' 03 whereas the maximum (2.8 µg/l) in May' 03. These values showed that the distribution of POC was not uniform at the study site and a marked fluctuation of 2.3 µg/l was observed during different months. However, these variations in POC were not effected the infectious activity of mussel hydrolysate.

The chlorophyll a content of the surface water was varied from 1.10 to 3.45 µg/l with a variation of 2.35 µg/l. Minimum values (1.10 µg/l) were recorded during April' 03 and maximum (3.45 µg/l) in February' 03 (Fig. 7.7). Similarly suspended solids values were ranged between 0.05 and 1.2 µg/l with a variation of 1.15 µg/l. Minimum suspended solids were recorded during March and April' 03 (fair season) and maximum in September' 02 (Fig. 7.8). The values of chlorophyll a content and suspended solids showed that the environment of the study site was uniform. Effects of these parameters on the bioactivity of the mussel hydrolysate were also not found significant.

The results obtained in haem-agglutination reaction are presented in Table 7.1. The analysis of variance was performed on the data for different environmental parameters and with the results of haem-agglutination reaction during different months. No significant correlation observed between infectious activity of the mussel hydrolysate prepared during different months and environmental parameters ($p>0.05$) was observed in the present study.

DISCUSSION

Environmental parameters such as salinity, temperature, pH, dissolved oxygen, particulate organic carbon have been reported to play a significant role on the metabolic activity of marine animals (Kutty *et al*, 1971; Gaudy and Sloane, 1981; Chen and Lin, 1985; Janakiram *et al.*, 1985 and Diwan *et al.*, 1989). Respiratory metabolism is considered to be an important biological process especially for the aquatic animals, which helps in assessing the oxygen requirement of the organism under different environmental conditions. It is also considered to be the best indicator for evaluating the energetic expenditure, precisely in the process of the osmotic regulation of any aquatic organism. In *P. monodon* and *P. stylirostris* the influence of salinity on the respiratory metabolism has been documented in detail where an increase in respiratory metabolism was observed at low salinities (Gaudy and Sloane, 1981). Attempts have also been made by several workers to demonstrate the effect of temperature on the physiological behaviour of the larvae of marine animals (Costlow *et al.* 1962; Young and Hazlett, 1978 and Moreira *et al.*, 1980). The normal respiratory metabolism in these organisms was at pH 6 and 7 whereas significant variation was observed at pH 5, 8 and 9.

The information on the effect of various environmental parameters on the infectious activity of extract prepared from aquatic animals is meagre. However, salinity has been reported to play a significant role on the biological activity of the horseshoe crab. The capability of detecting pyrogens by the amoebocyte lysate prepared from the blood of the horseshoe crab has been observed to be reduced significantly at lower salinity (Chatterji, 1994).

Many workers have reported the influence of salinity on the metabolic changes in higher marine invertebrates with their acclimation and survival ability at different degrees of salinity. Roche and Bogé (1996) reported changes in antioxidant enzymes in the red blood cells of sea bass (*Dicentrarchus labrax*) subjected to hypo-osmotic shock. Osmoregulation undoubtedly is a greater energy expense and caused by metabolic activation, which consequently increase the ROS formation. A direct relationship has been found between metabolic intensity and activation of oxidative enzymes in higher invertebrates (Wilhelm Filho *et al.*, 1993).

It has been reported that as a consequence of metabolic activity due to change in environmental conditions, reactive oxygen species (ROS) are continuously produced and acted as strong oxidants. Usually, as a defence mechanism, a large repertory of antioxidant enzymes, in addition to small antioxidant molecules are produced by the cell. Superoxide Dismutase (SOD), which causes the dismutation of O_2^- to H_2O_2, catalase (CAT), glutathione peroxidase (GPX) and converts H_2O_2 to H_2O, are the most important antioxidant enzymes found in all marine animals. Their activities differ among the organs and tissues depending upon environmental factors and other ecological conditions (Winston and Di Giulio, 1991; Roche and Bogé, 1996). When antioxidant defences are inadequate to combat the action of the ROS, the result has been reported to be oxidative stress (Winston and Di Giulio, 1991). The formation of ROS has been found to increase in response to different internal or external medium or environmental conditions, whereupon oxidative alterations occur in the cellular constituents. One of the alterations is increased the lipid per-oxidation as a consequence of the oxidation of the lipid constituents of cell membranes (Roche and Bogé, 1996).

A possible repercussion that osmoregulatory processes could induce the classical indicators of stress (e.g. cortisol, glycaemia and haemoglobin) with gradual increase in environmental salinity and maintained for 20 days at a constant concentration of 35% was studied in *A. naccarii* by Martínez-Álvarez *et al.*, (2002). The activities of SOD, CAT and GPX, as well as lipid-peroxidation levels in blood (plasma and red blood cells), liver and heart, were monitored by these workers. A decline has been reported in total plasma proteins with increasing salinity that could be due to high osmoregulatory energy demand (Usher *et al.*, 1991; Plaut, 1998). In *A. naccarii*, blood (plasma and red blood cells), antioxidant defences (SOD, CAT and GPX activities) have the ability to strengthen under increasing environmental salinity (Martínez-Álvarez *et al.*, 2002). The increase of these enzyme activities in red blood cells could explain the decline in lipid-peroxidation values and the stability of these values despite changing salinity conditions. It seems that the activity of these enzymes in the plasma is not sufficient to avoid a certain degree of lipid oxidation at 35 ppt salinity.

In addition, SOD and CAT have been found to decrease significantly in the liver with increase in salinity conditions (Martínez-Álvarez *et al.*, 2002). The trend when not accompanied by lipid peroxidation, would indicate a certain alteration of the cell metabolism rather than a state of oxidative stress. The enzymatic activities have been expressed as a function of the protein content and the relative protein content of the liver increased with increasing environmental salinity (Martínez-Álvarez *et al.*, 2002). This increase may result from a decrease in fatty acid content in response to a deficit in energy intake, as discussed above, or to reduced body-water content. Thus, the increase in hepatic protein is a possible reason for the depressed SOD and CAT activities. Roche and Bogé (1996) also reported the changes in enzyme activities (SOD, CAT and peroxidase) in *D. labrax* during replacement of seawater (37 ppt) with freshwater (5 ppt). The decrease in salinity stimulates SOD and CAT activities by hypo-osmotic shock (Roche and Bogé, 1996). However, this phenomenon was not observed in any of the present studies, which could determine the parameters of oxidative stress under conditions of hyper-osmotic stress.

Environmental disturbance has been considered a potential source of stress as it prompted a number of responses in the animal to deal with the physiological changes triggered by extrenal changes (Cataldi *et al.*, 1995). These responses can be detected in vertebrates in the form of changes in hormonal or substrate concentrations in the plasma or alterations in erythrocyte parameters, such as cell volume or enzymatic activities (Donaldson, 1981). The internal perturbation of the organisms either directly or as a result of alterations of the environment, overwhelms the physiological mechanisms of the animal to response for adaptation to new conditions. However, in lower vertebrates like bivalves, the detection in variation of enzymatic activities in relation to change in environmental conditions is rather difficult. Dissolved oxygen is an important environmental constituent and the scarcity of it in the environment results in various phenotypic and physiological changes in the animal for their survival in a particular environment. This is because of the tremendous stress occurred on account of the depletion of oxygen. Surprisingly, most of the work carried out on this aspect is limited to only higher vertebrate groups rather than bivalves and other invertebrates. In the present study, no significant observation was made which could support the findings of the above investigations.

Table 7.1: Neutralization reaction on Chicken embryo (VCA) with samples of mussel hydrolysate during different months and virus A/Mississippi/85/H_3N_2.

		Virus dilution (A/Mississippi/85/H_3N_2)							
Months	Sample	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	EID_{50}	Difference
Sept' 02	Control	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	- - - -	6.50*	-
	Sample	+ + + +	+ + + +	+ - - -	- - - -	- - - -	- - - -	3.75*	2.75
Oct.	Control	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ - - -	6.75*	-
	Sample	+ + + +	+ + + +	+ + - -	- - - -	- - - -	- - - -	4.00*	2.75
Nov.	Control	+ + + +	+ + + +	+ + + +	+ + + +	+ + + -	- - - -	6.25*	-
	Sample	+ + + +	+ + + +	+ - - -	- - - -	- - - -	- - - -	3.75*	2.50
Dec.	Control	+ + + +	+ + + +	+ + + +	+ + + +	- - - -	- - - -	5.50*	-
	Sample	+ + + +	+ - - -	- - - -	- - - -	- - - -	- - - -	2.75*	2.75
Jan.' 03	Control	+ + + +	+ + + +	+ + + +	+ + + +	+ + - -	- - - -	6.00*	-
	Sample	+ + + +	+ + - -	- - - -	- - - -	- - - -	- - - -	3.00*	3.00
Feb.	Control	+ + + +	+ + + +	+ + + +	+ + + +	+ + - -	+ - - -	6.25*	-
	Sample	+ + + +	+ + + +	- - - -	+ - - -	- - - -	- - - -	3.75*	2.50
March	Control	+ + + +	+ + + +	+ + + +	+ + + +	+ - - -	- - - -	5.75*	-
	Sample	+ + + +	- - - -	- - - -	- - - -	- - - -	- - - -	2.50*	2.50
April	Control	+ + + +	+ + + +	+ + + +	+ + + +	+ - - +	- + - -	6.25*	-
	Sample	+ + + -	- - - -	- - - -	- - - -	- - - -	- - - -	2.25*	3.00
May	Control	+ + + +	+ + + +	+ + + +	+ + + +	+ + - +	- - - -	6.25*	-
	Sample	+ + + +	+ + + -	- - - -	- - - -	- - - -	- - - -	3.25*	3.00
June	Control	+ + + +	+ + + +	+ + + +	+ + + +	- - - -	- - - -	5.50*	-
	Sample	+ + + +	- - - -	- - - -	- - - -	- - - -	- - - -	2.50*	3.00

+, Shows presence of virus; -, Shows absence of virus; * 0.5 added to each values

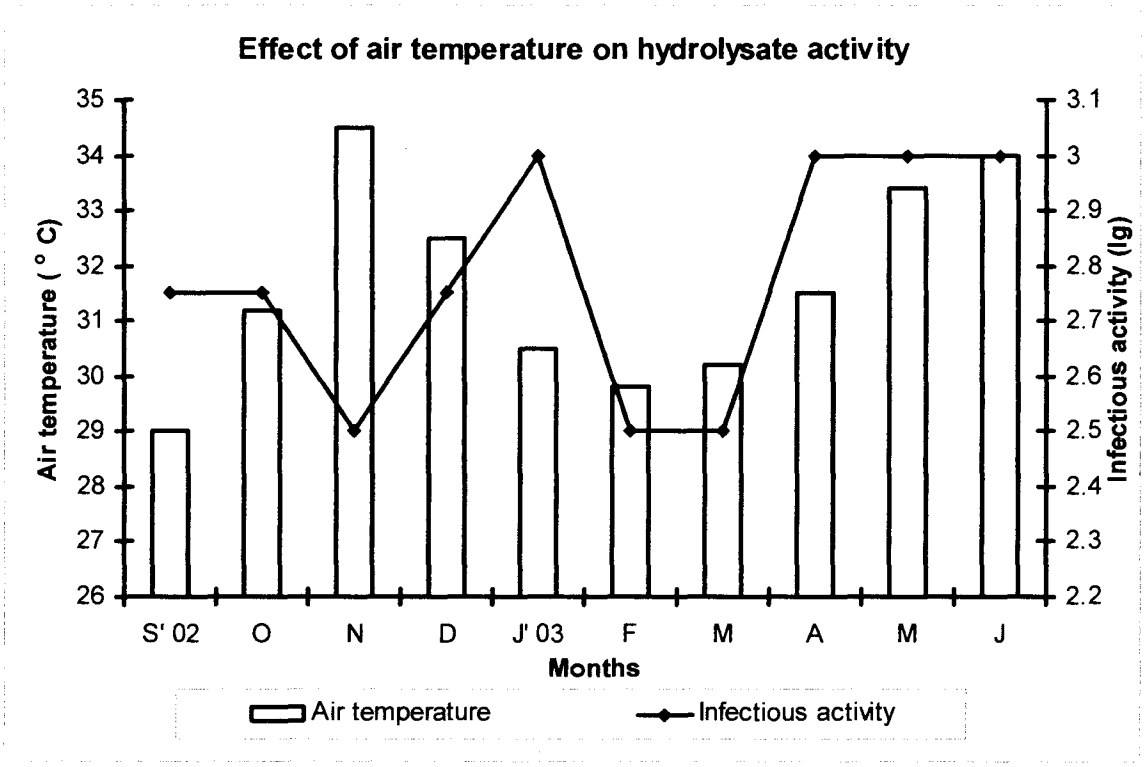


Fig. 7.1. Effect of air temperature on infectious activity of the mussel hydrolysate

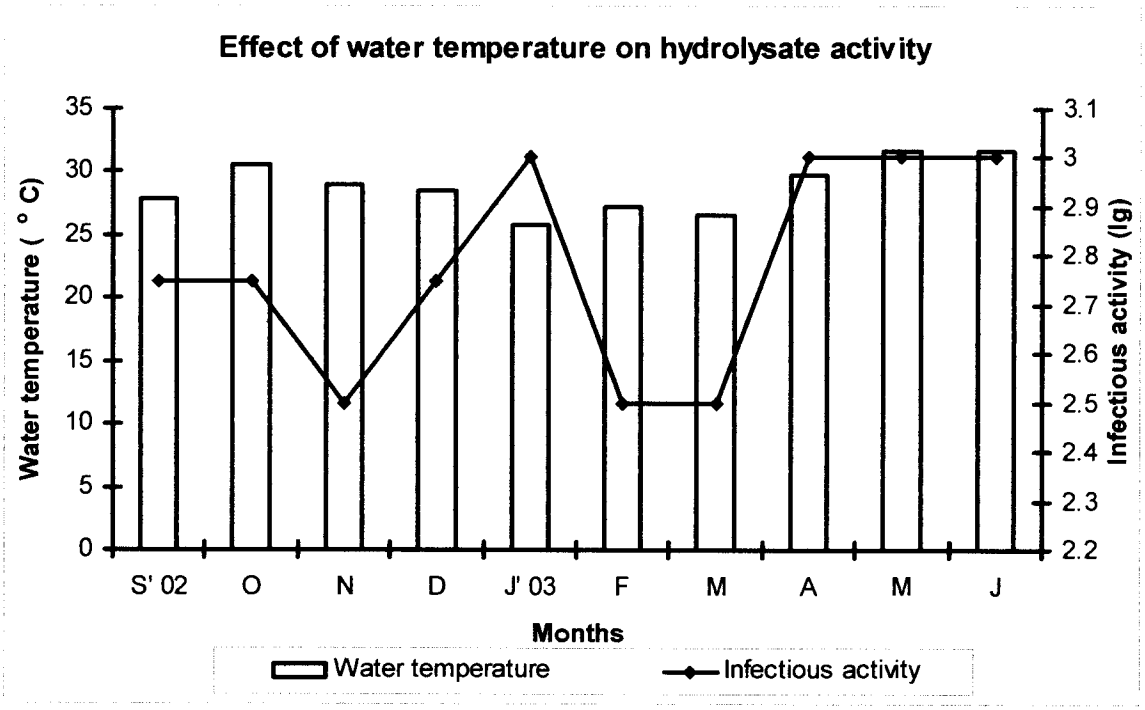


Fig. 7.2. Effect of water temperature on infectious activity of the mussel hydrolysate

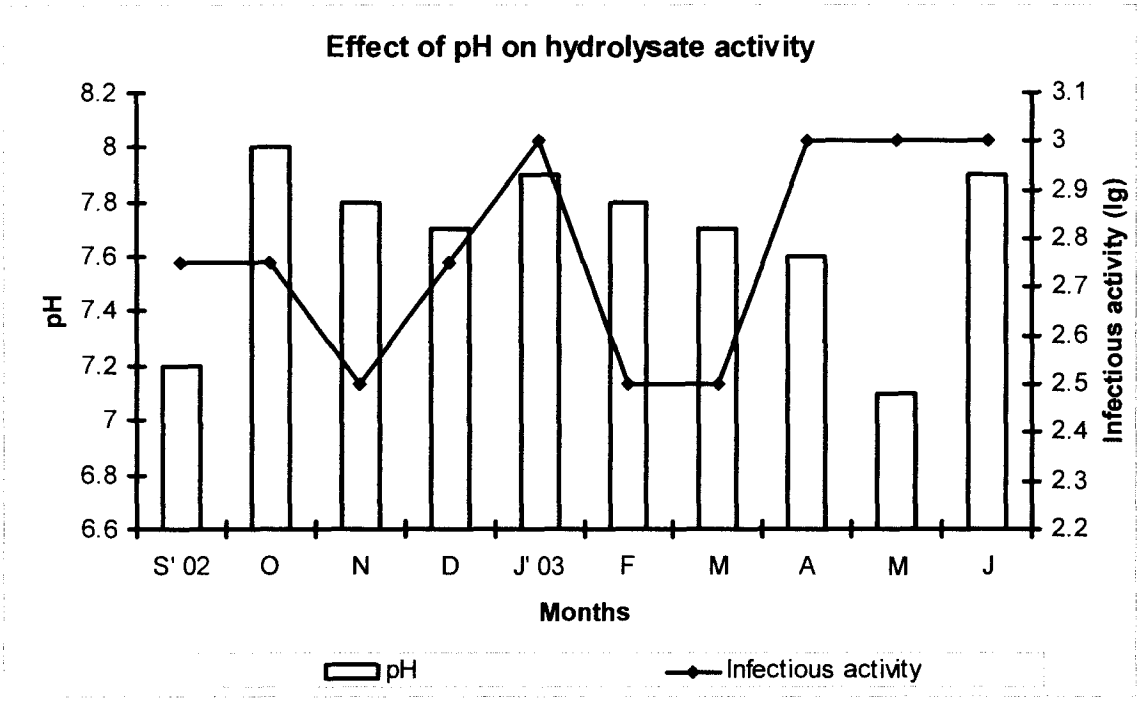


Fig. 7.3. Effect of pH on infectious activity of the mussel hydrolysate

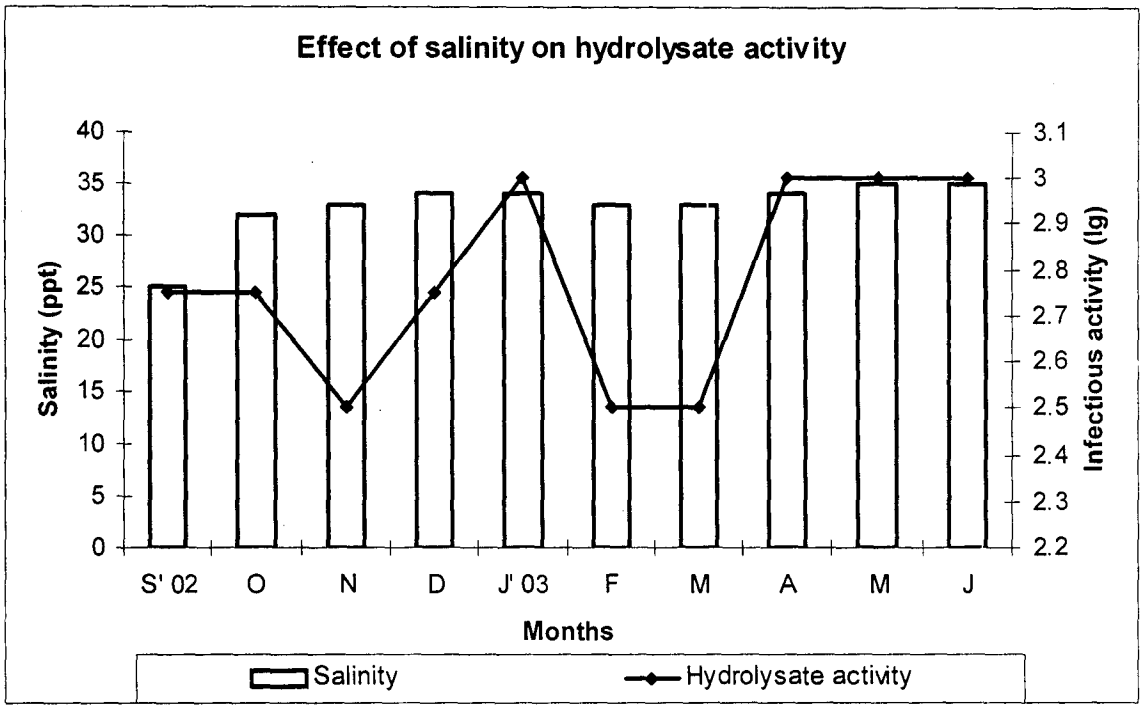


Fig. 7.4. Effect of salinity on infectious activity of the mussel hydrolysate

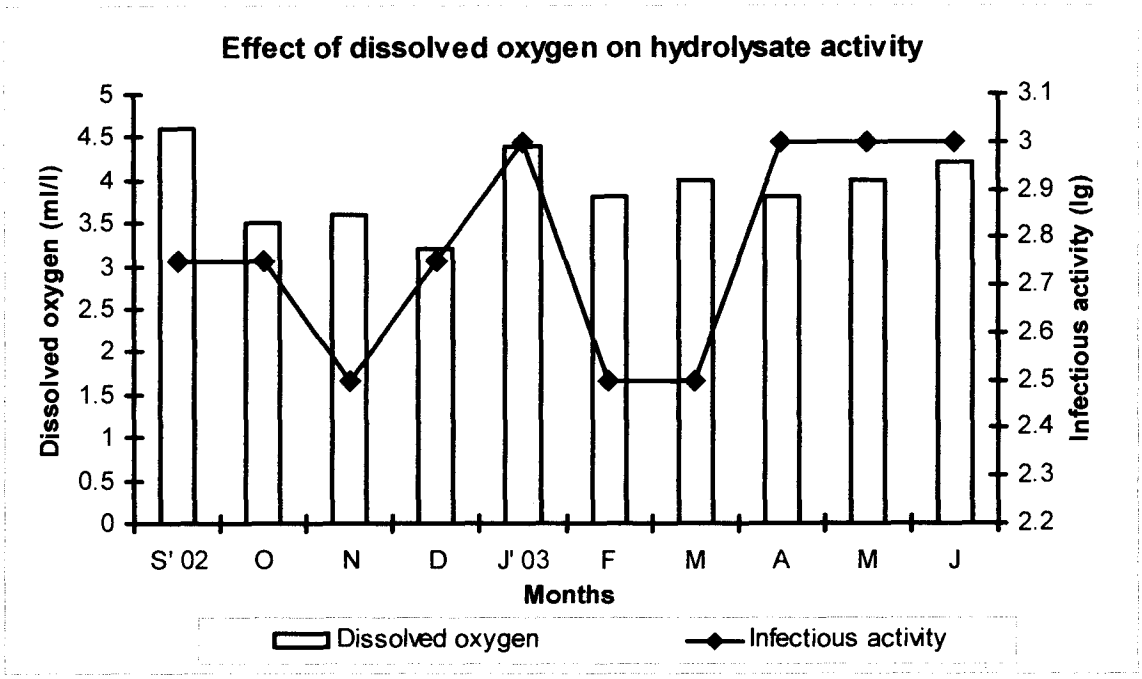


Fig. 7.5. Effect of dissolved oxygen on infectious activity of the mussel hydrolysate

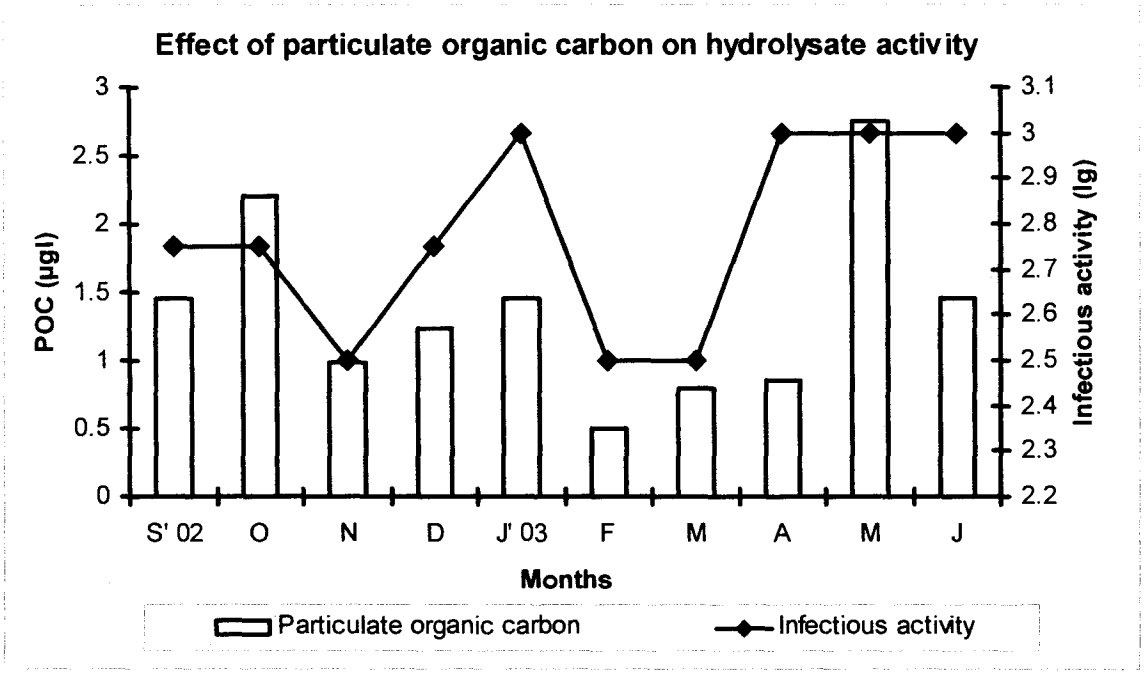


Fig. 7.6. Effect of particulate organic carbon on infectious activity of the mussel hydrolysate

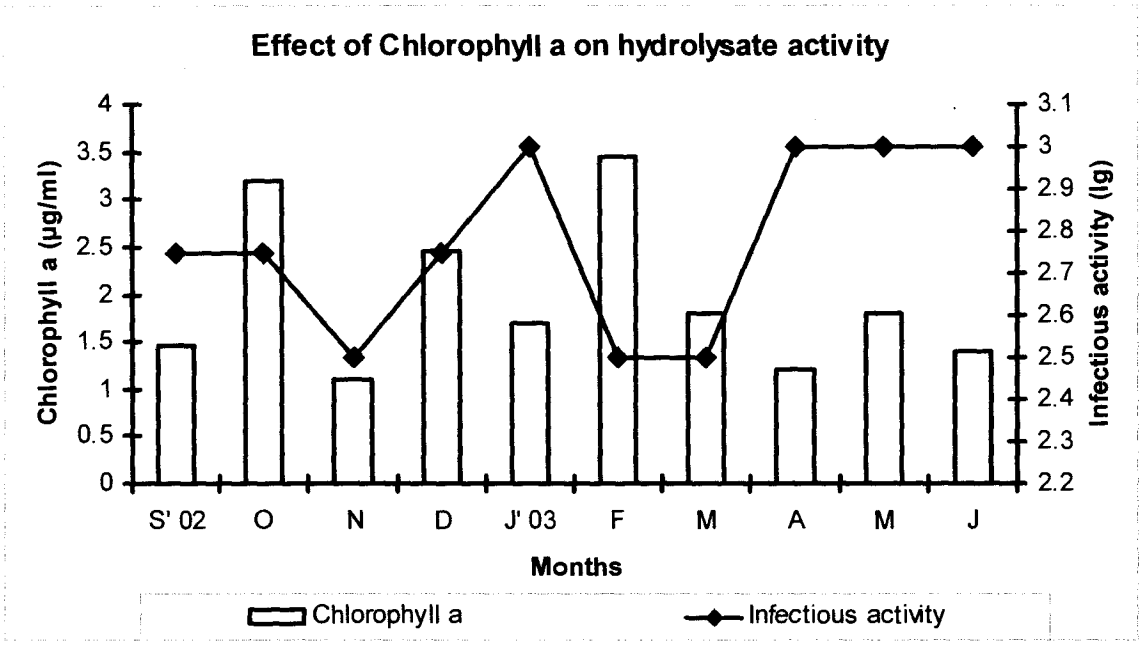


Fig. 7.7. Effect of Chlorophyll a on infectious activity of the mussel hydrolysate

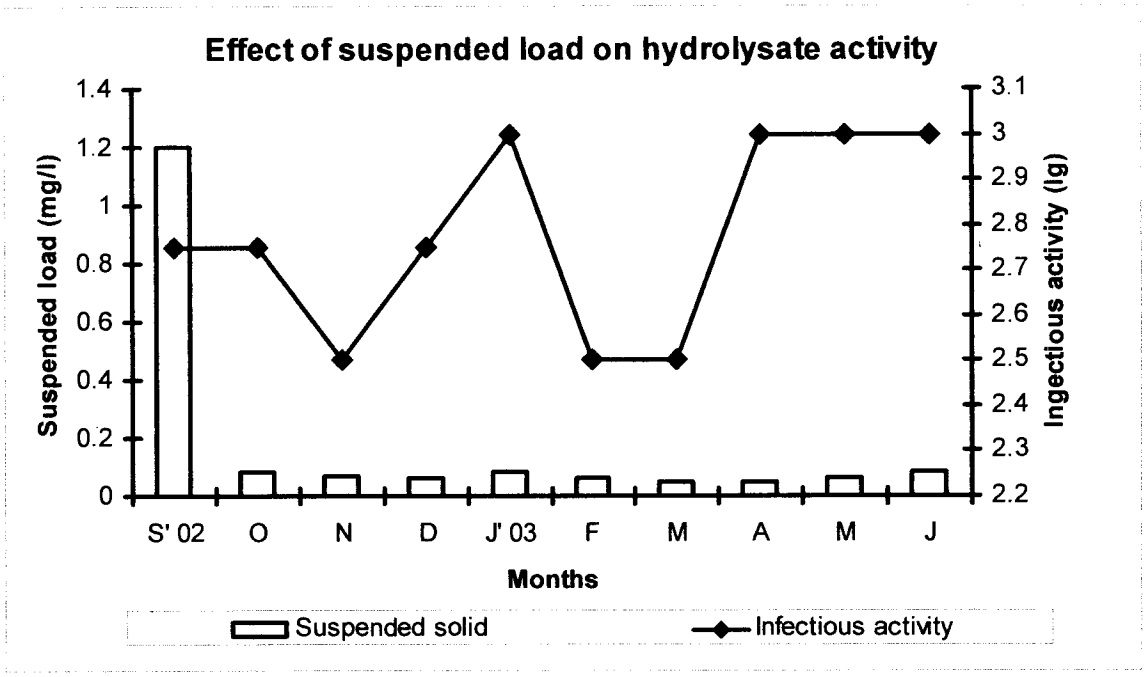


Fig. 7.8. Effect of suspended load on infectious activity of the mussel hydrolysate

CHAPTER 8

Biochemical composition of tissue of green mussel and its relation with infectious activity

In India, coastal and estuarine areas sustain vast resources of molluscs. Marine bivalve molluscs, a potential source of valuable protein, carbohydrates and minerals, are abundantly available in Indian waters. Among them, edible mussels are having great potential because of their high productivity. These bivalves have been exploited by the people of the coastal areas from time immemorial for food and also for their shells. An understanding of its nutritional aspects can lead to better utilization of the resources. Seasonal cycle of the variation of the biochemical constituents like protein, carbohydrates and lipid are generally attributed to the complex interaction between environmental parameters. Food availability, growth and reproductive activities are the most important biological parameters (Bayne, 1976; Gabbot, 1973; 1976; Sastry, 1979). The study of variation in energy in the form of protein, glycogen and lipid would help in understanding the ecology and overall economy of the species. A sound knowledge of variations in biochemical composition in different stages of growth is essential and is helpful for the exploitation of bivalves at time when their nutritional value is higher.

In recent years much emphasis have been focused on the nutritive aspects of marine organisms and among these, molluscs are preferred more as they have high nutritive value and are also easy to cultivate on a commercial scale (Rivonker, 1991). Research on biochemical composition of the marine molluscs during 1950-60, highlights the pioneering work in this field (Korringa, 1956; Baird, 1958; Dare and Edwards, 1975). The change in body weight has been reported mainly due to changes in carbohydrate content (Dare and Edwards, 1975). In India, early studies were carried out by Venkataraman and Chari (1951) who reported that high lipid content in mussel tissue was found to coincide with intensive feeding. Studies on the biochemical composition of the mussel were carried out by Williams (1969) who documented that seasonal change in lipid content of *Mytilus edulis* showed an inverse correlation with glycogen content. A review of a new approach to study the biochemical composition of the molluscs was presented by Giese (1969).

The physiological changes in *Mytilus edulis* induced by temperature, nutritive stress and the biochemical ecology of molluscs have been studied in temperate waters by Gilles (1972), Bayne (1973) and Gabbot and Bayne (1973). They concluded that temperature influenced biochemical constituents to a certain extent. Pieters *et al.* (1979) studied the changes in glycogen, protein, total lipid and energy metabolism in *Mytilus edulis* in relation to environmental factors and the reproductive cycle. Seasonal changes in flesh weight and biochemical composition of the scallop, *Chlamys opercularis* have been conducted by Taylor and Venn (1979). They found that the seasonal cycle of weight and biochemical composition of the gonad were closely followed the spawning cycle of the animal.

A detailed study with reference to energy metabolism and gametogenesis has been carried out by Zandee *et al.* (1980) who demonstrated that no seasonal variation in free amino acids occurred in total tissue and different organs. Shafee (1981) carried out studies on the seasonal changes in the biochemical composition and calorific content of the Black scallop (*Chlamys varia* L) collected from Louveoc, Bay of Brest. The main objective of his research was to understand the relationship between reproductive cycle, storage and utilization of food reserves. Walter (1982) conducted studies on growth and reproduction in tropical mussel, *Perna viridis.* He found that though the spawning of the animal was protracted, there was no consistency in reproductive activity. Recently Deslous- Paoli and Heral (1988) carried out studies on biochemical composition and energy value of *Crassostrea gigas* (Thuberg) cultured in Bay of Marennes Oleron and opined that the quality of food available during phytoplankton bloom was linked with reserve of energy mainly as glycogen.

In the present study, an attempt was made to see the effect of changes in biochemical composition of the tissue of green mussel on the bioactivity of the mussel hydrolysate. The mussel hyrdolysate was prepared in different seasons and its infectious activity was assessed on *in vitro* model using viral strain A/Mississippi/85/H_3N_2 at the Pasteur Institute, St' Petersburg, Russia.

MATERIALS AND METHODS

The samples for the present investigations were collected from Dona Paula Bay during September' 2002 to June' 2003. Total weight of each specimen was determined to the accuracy of 0.1 mg after removal of excess water. Wet tissues of the mussel were used for the estimation of different biochemical constituents. Wet weight was measured after washing the tissue with distilled water and removing the excess water with filter paper. In order to determine the dry weight, the weighed samples were dried at 60-80° C until a stable weight was attained. The water content was calculated by the following formula:

$$\frac{\text{Wet weight - Dry weight x 100}}{\text{Wet weight}} = \text{percent wet weight}$$

The dried tissue samples were used for biochemical analysis. Dried samples were powdered using mortar and pestle and used for protein, carbohydrate and lipid analysis.

Estimation of Protein by Folin's Lowry method

Dried tissue of green mussel, approximately 10 mg, was taken into an ordinary test tube with 1 ml of 1N NaOH solution. The test tube was kept in warm water at 80° C for about half an hour. This was followed by an addition of 1 ml of 1 N HCl and the material was centrifuged at 2000 rpm for 10 minutes. Supernatant solution of 1 ml was taken into another test tube and 2.5 ml of mixed reagent was added to it. Folin's reagent (0.5 ml at a ratio of 1:2 with distilled water) was then added and the material was allowed to stand for 30 minutes for the development of complete colour. The optical density of the sample was recorded at 750 nm using a spectrophotometer and protein estimated with the help of standard graph using Bovine Serum Albumin as standard (Herbert *et al.*, 1971).

Reagents

1. 1 N NaOH (40 g of dry NaOH in 1000 ml of double distilled water).
2. 0.1 N NaOH (4 g of NaOH in 1000 ml of double distilled water).
3. Mixed Reagent: (Alkaline Copper Tartrate Reagent)
   a) Reagent A: 2% Na_2Co_3 in 0.1 N NaOH
   b) Reagent B: 1% Sodium Potassium Tartrate in 0.5% $CuSO_4$

The mixed reagent was prepared by mixing 50 ml of reagent A and 1 ml of reagent B

4. Folin's Reagent:
   Folin's phenol reagent was diluted with distilled water at the ratio of 1: 2

Estimation of Carbohydrates

The carbohydrate was estimated by phenol-sulphuric acid method as described by Dubois *et al.*, 1956) and subsequently modified by Hitchcock (1971). For this, 10 mg of dried mussel tissue was taken in a clean test tube with 2 ml of 80% H_2SO_4. The material was kept for 16-18 hours for digestion. This was followed by addition of 2 ml of 5% phenol and 10 ml of Conc. H_2SO_4. The test tube was then allowed to stand for 2 hours that was followed by centrifugation of the samples. The absorbance of the supernatant was measured at 490 nm. Standard curve was plotted with the help of D-Glucose (Analar grade). All concentrations were expressed as mg/g dry weight of mussel tissue.

Estimation of total lipid

Total lipid was determined by the Parsons *et al.* (1984) method of oxidation by acid dichromate. The oxidation reaction is followed by the decrease in the dichromate colour. Extinction of the reaction medium showed an inverse relationship based on the decrease of the dichromate colour. A known amount of the dried mussel tissue (10 mg) was placed in a homogenizer with 8 ml chloroform-methanol mixture (2:1). The above extract was mixed with sodium chloride (0.2 ml of 0.9 N) in a test tube and was then capped with non-adsorbent cotton. This was allowed to equilibrate overnight at 4-5° C. It was then transferred to a separating funnel and the lower layer was drained into a clean test tube. After adding Conc H_2So_4 (0.5 ml), the solution was warmed for ten minutes in

a boiling water bath, cooled and phosphovanillin reagent (5 ml) was added. After shaking properly the solution, optical density was measured at 520 nm. Standard graph was prepared using cholesterol to calculate the lipid concentration. Lipid content thus obtained was expressed as mg of lipid per gram of the tissue.

The methodology of preparing reagents required for screening biological activities were the same as described in Chapter 7 (Table 7.1). The relationship between biological activity of mussel hydrolysate in terms of infectious activity (Ig) and biochemical composition was examined using Pearson's correlation coefficient (Legendre and Legendre, 1983).

RESULTS

Fluctuations in the moisture content during different months in mussel tissue represented as percentage are given in Figure 8.1. Moisture content in the tissue was between 64.0 and 84.1%. Maximum moisture content (84.1%) was recorded in June' 2003 whereas minimum (64.0%) in April' 2003 (Fig. 8.1). Otherwise in all other months, not much variation in moisture values was observed in the present study. This showed that the fluctuation in moisture content was only to the tune of 20.1% during the study period. In the present study, high moisture content in the tissue was examined during pre-monsoon period.

The protein values were low during September' 2002 whereas it was maximum in March' 2003 (Fig. 8.2). However, in other months, there was no major fluctuation in the protein value. The low protein values just immediately after the monsoon months were in coincidence of maturation and spawning period of the mussel.

Maximum carbohydrate content values were recorded during October' 2002 which was in coincidence with spawning period of the green mussel. The minimum value of carbohydrate was recorded in September' 2002. These values varied from 24 to 70 mg/g dry wt (Fig. 8.3). In our studies, not much variation in the carbohydrate content was observed except a little higher value during post monsoon period.

The lowest value of lipid content was observed in March' 2003 and the variation in lipid value was in the range of 90 to 190 mg/g dry wt (Fig. 8.4). This showed that the variation in the lipid content during the study period was relatively high as compared to other biochemical parameters. The maximum value of lipid in October' 2002 was just after the spawning period when the carbohydrate values were also observed to be high.

Analysis of variance was performed on the data of mean infectious activity calculated in different months with biochemical parameters. There was no significant correlation ($p>005$) between the infectious activity and the biochemical composition of the tissue of green mussel (Figs 8.1-8.4).

The results obtained during present studies further confirmed that neither the influence of environmental parameters nor biochemical composition of the mussel tissue play any significant role in the fluctuating the infectious activity of mussel hydrolysate.

DISCUSSION

Studies on the biochemical composition of different species of bivalves have received the attention of scientific workers in several parts of the world because of their importance as food and their role in the economy. Information on biochemical composition of the mussel tissue is important as it showed directly the nutritive value of the mussel. Most of the works on the biochemical composition of marine bivalves have been concerned with the gross changes in protein, lipid and carbohydrate contents.

Several workers have estimated the organic constituents in the whole tissue of many bivalves like *Donax vittatus* (Ansell, 1972), *D. spiculum* (Ansell *et al.*, 1973), *Donax cuneatus* (Nagabhushanam and Talikhedkar, 1977), *Donax trunculus* (Ansell, *et al.*, 1980), *Meretrix casta*, *Sanguinolaria diphos* (Wafar, 1974), *Meretrix meretrix* (Jayabal and Kalyani, 1986), *Abra alba* (Ansell, 1974a), *Chlamys septemradiata* (Ansell, 1974b), *Nucula montagui* (Ansell, 1975), *Muscullista arcuatula* (George and Nair, 1975), *Villorita cyprinoidis, V. cochinensis* (Nair and Shynamma, 1975a, Lakshmanan and Nambisan, 1980, Ansari *et al.*, 1981), *Mytillus viridis* (Wafar *et al.*, 1976 and Nagabhushanam and Mane, 1978), *Perna viridis* (Shafee, 1978), *Crassostrea gigas*, *Ostrea edulis* (Mann, 1979), *Crassostrea casta* (Lakshmanan and Nambisan, 1980), *Tapes decussates* and *T. philippinarum* (Beninger and Lucas, 1984), *Mytillus galloprovincialis* (Bressan and Marin, 1985).

Nagabhushanam and Mane (1978) studied the reproduction and seasonal variation in biochemical composition of estuarine mussel, *Mytilus viridis* from Ratnagiri region of West coast of India. They suggested that the level of major biochemical constituents such as protein, fat and glycogen showed correlation with reproductive cycles in mussels. The total caloric content of the cultured mussels on floating raft has been reported to be higher than mussels collected from the natural beds (Qasim *et. al.*, 1977; Rivonker, 1991). Shafee (1978) conducted a detailed study on the variation in the biochemical composition of the green mussel, *Perna viridis* L. from Ennor estuary in Madras. The maximum calorific values were in coincidence with peak protein values.

Seasonal changes in biochemical constituents in edible molluscs were also conducted by some other workers along the Indian coast (Suryanarayanan and Alexander, 1972; Wafar *et al.*, 1976). These workers have suggested that high values of caloric content were during pre-spawning seasons. More recently, Mohan and Kalyani (1989) conducted a study on seasonal variation in biochemical composition of the green mussel, *Perna viridis* L. where males and females did not show any difference in the biochemical constituents.

Salinity plays a significant role on increase and decrease of the moisture content of the tissue (Rivonker, 1991). During low saline conditions in monsoon seasons, the moisture increased to 92.5% in oysters (Galtsoff, 1964) and 90% in green mussel (Rivonker, 1991). A similar observation was made in the present study when higher values of moisture (84%) was recorded in June' 2003. The higher values of protein, carbohydrate and lipid were recorded in fair season when the availability of food material in the environment was high. The trend of these values in the present study was same as reported by other workers.

It has been reported that reproductive cycle plays an important role on biochemical composition of the bivalve tissues (Gabbot, 1976; Rivonker, 1991). The lower values in protein, carbohydrate and lipid were just after the monsoon months, which were in coincidence with spawning period of the mussels. In the present study, samples of green mussels were not available during monsoon months due to bad weather conditions.

In the present study, however, interpretations made from the statistical analysis highlighted that there was no definite correlation found between the moisture, protein, carbohydrate and lipid content of the tissue with the infectious activity of mussel hydrolyate. This shows that biochemical parameters and seasons have no impact on the infectious activity of mussel hyrdolysate. However, the biological activity of mussel hyrdolysate in terms of infectious activity was found different when a comparison was made between the hyrdolysate prepared from blue mussels collected from the black sea in Russian waters with Indian green mussels (Chatterji *et al*, 2002).

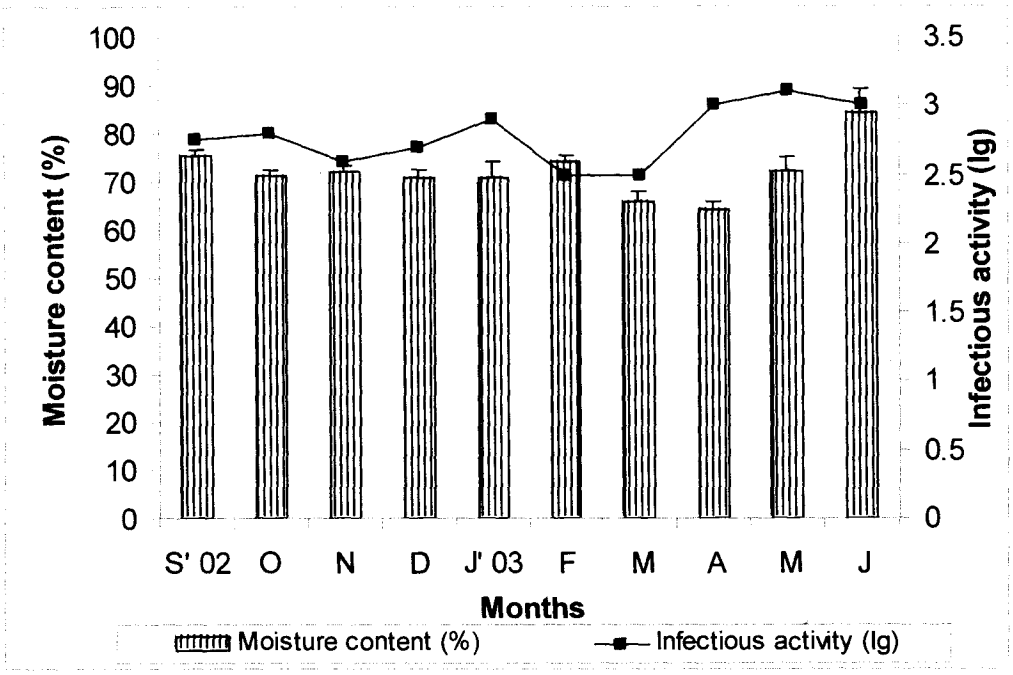


Fig. 8.1. Relationship between moisture content and infectious activity

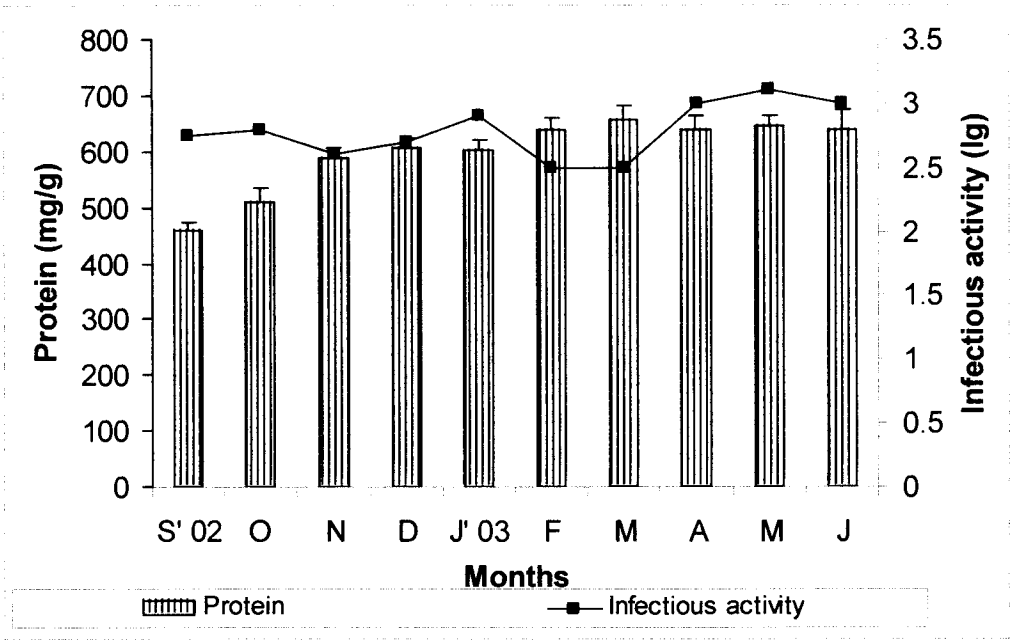


Fig. 8.2. Relationship between protein content and infectious activity

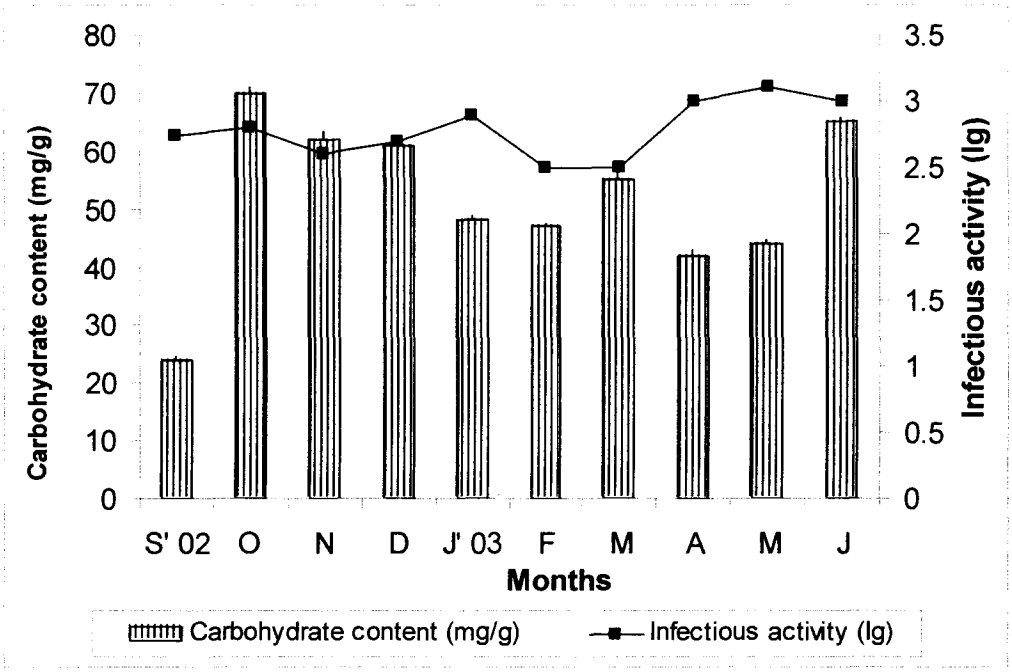


Fig. 8.3. Relationship between carbohydrate content and infectious activity

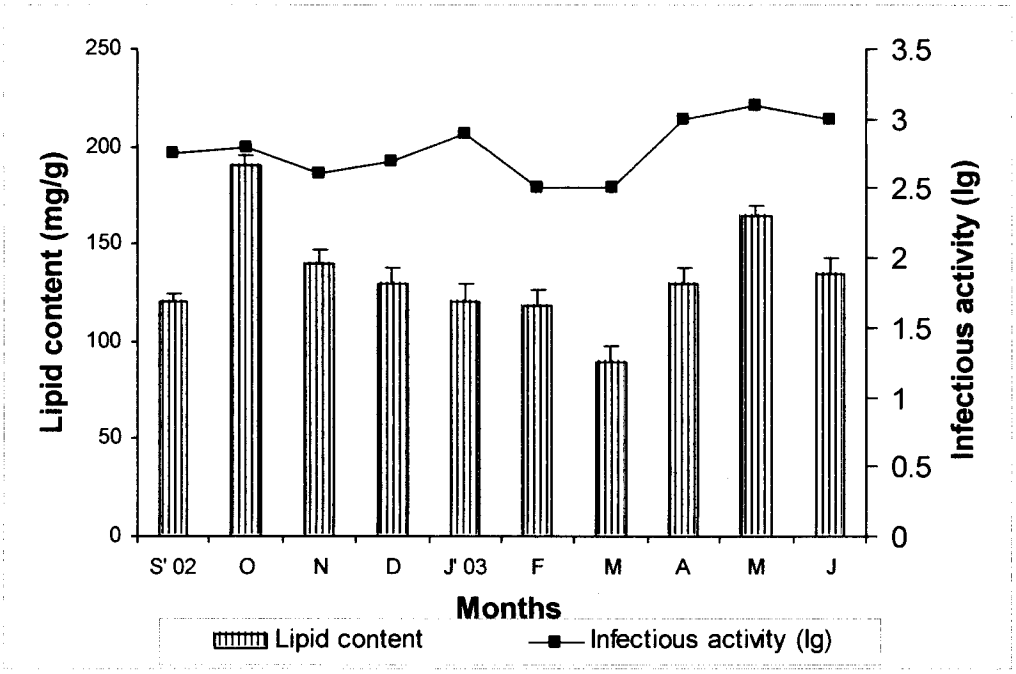


Fig. 8.4. Relationship between lipid content and infectious activity

SUMMARY

The present study was carried out to investigate the radioprotective activity of mussel hydrolysate (MH) from Indian green mussel, *Perna viridis (L)*. Mussel hydrolysate was screened for radioprotective activity in *E. coli* and *Saccharomyces cerevisiae* by means of dose response survival curves. The mechanism of radioprotection of mussel hydrolysate was studied by means of free radical scavenging activity, antioxidant enzyme activities and also by protection of DNA damages in plasmid and in mice lymphocytes by comet assay. A study was also carried out to find the effect of different environmental and biochemical parameters to know their effects on the sensitivity on the biological activity of the mussel hydrolysate.

- Dose response survival study in *E. coli* cells showed that both crude and aqueous mussel hydrolysate have radioprotective activity by increasing the D_{10} value. Crude mussel hydrolysate of 10% concentration significantly increased the D_{10} value from 67 Gy of control cells to 156 Gy of cells treated with hydrolysate. The aqueous extract of 5% concentration increased the D_{10} value to 115 Gy. In another study, it was found that radioprotective activity of crude mussel hydrolysate increased when the concentration of the extract increased from 3 to 10%. There was no further increse in the radioprotective activity with increase in concentration of MH. Crude extract of 10% concentration showed nearly 27 times more protection than the control irradiated sample whereas, aqueous extract of 10% concentration showed only 3 fold protection. However, cysteine of 0.5% concentration showed nearly 189 times more than the control irradiated cells.

- Radioprotective studies in yeast *Saccharomyces cerevisiae* cells showed that crude mussel hydrolysate significantly enhanced the survival of yeast cells. The percentage of survival of yeast cells was decreased from 65.98 to 3.50% when the cells were irradiated with radiation dose from 150 to 1050 Gy. The D37 value of 355 Gy was obtained for the control-irradiated sample. On the contrary, when the yeast cells were incubated with mussel hydrolysate (concentration: 0.05 g/ml) and irradiated with radiation dose from 150 to 1050 Gy, the D_{37} value was 415 Gy showing significant survival of yeast cells indicating its radioprotective activity. When yeast cells were incubated with 1 to 5 g/ml of mussel hydrolysate and irradiated with 250 Gy, there was a significant protection of cells from 31.5 to

48.46%. Maximum radioprotection activity was found with mussel hydrolysate at a concentration of 0.05 g/ml.

- Mussel hydrolysate showed significant scavenging activity of free radicals when free radicals were produced both by radiation and chemically induced by means of Azo-bis. Mussel hydrolysate showed significant concentration dependent scavenging activity of free radicals with increasing concentration of mussel hydrolysate (0.1 to 10 mg/ml) in mice lymphocyte cells. At 0.1 mg/ml of mussel hydrolyzate, the scavenging activity was 48.52% when a radiation dose of 2 Gy was given. However, scavenging activity was 51.12% when ROS was induced chemically by Azo-bis. Similarly, the scavenging activity was increased to 80.26% at a concentration of 10 mg/ml of mussel hydrolysate at 2 Gy dose of radiation as compared to 80.77% with Azo-bis. Not much variation of free radical scavenging activity was observed at both the concentrations of 0.1 and 10 mg/ml of mussel hydrolysate using radiation induced ROS and chemically induced by Azo-bis.

- Mussel hydrolysate showed significant protection of plasmid DNA from γ-radiation induced strand breaks in *in vitro* experiments. Mussel hydrolysate (25 mg/ml) showed a significant protection of plasmid DNA from damages generated upon exposure with increasing doses of γ-radiation from 30 to 360 Gy. An exposure of 120 Gy γ-radiation was found to reduce the supercoiled form of DNA (**ccc**) approximately up to 19% as compared to un-irradiated control (100%) that indicated a significant reduction of **ccc** form of DNA due to strand breaks. Mussel hydrolysate concentration of 5 to 50 mg/ml significantly reduced the disappearance of **ccc** form of DNA in a concentration dependent manner. In comparison with irradiated control, the presence of 25 mg/ml of mussel hydrolysate during radiation was found to restore the **ccc** levels up to 81% whereas 50 mg/ml mussel hydrolysate restored the **ccc** levels up to 100%. Comet assay showed a significant reduction of DNA damage with 0.5 mg/ml of mussel hydrolysate in mice lymphocytes when exposed to gamma irradiation of 2 Gy. The number of cells having higher values for comet parameters such as tail length, tail moment and percent DNA in tail decreased significantly when the lymphocyte cells were irradiated with mussel hydrolysate. These results clearly

indicated that the mussel hydrolysate significantly reduced the γ radiation induced DNA damages in lymphocyte cells.

- Mussel hydrolysate concentrations of 0.25 mg/ml to 0.5 mg/ml significantly enhanced the activity of antioxidant enzymes catalase and superoxide dismutase in mice lymphocytes. An increase of 37% more catalase activity than the control cells was found when the lymphocyte cells were incubated for 24 hours with 0.5 mg/ml of mussel hydrolysate. Superoxide dismutase activity increased 28% than the control cells, when cells were treated with same concentration of mussel hydrolysate. This characteristic of mussel hydrolysate can also be attributed to its radioprotective properties.

- The effects of different environmental parameters such as temperature, salinity, dissolved oxygen, pH, particulate organic carbon, chlorophyll a and suspended load on the sensitivity of mussel hydrolysate in terms of infectious activity of mussel hydrolysate showed that there was no significant correlation observed between infectious activity of the mussel hydrolysate prepared during the study period and environmental parameters.

- There was no significant correlation was observed between the biochemical composition (protein, carbohydrate and lipid) of the tissue of green mussel and the sensitivity of the mussel hydrolysate. The results obtained during present study further confirmed that neither the influence of environmental parameters nor biochemical composition of the mussel tissue play any significant role in the fluctuating the infectious activity of mussel hydrolysate.

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