Introduction

Biological effects of ionizing radiation are attributed to the chemical changes in the biological molecules that originate with energy absorption. Cells and tissues succumb to radiation damage due to direct effect resulting from direct ionization of target molecules and due to indirect effect produced in the target molecule by reactions with the free radicals formed by aqueous radiolysis as the tissues contain 80% water (Nair *et al*., 2001). Cellular radiosensitivity is governed by various factors such as rate of cell proliferation, presence of various types of cellular DNA repair systems, antioxidant enzymes, sulfhydryl compounds, vitamins and chemicals. Variations in any of these factors could alter cell's response to ionizing radiation. In addition to this, the physiological state of cells, cell cycle stage as well as the oxygen status influences the radiosensitivity. Radiotherapy is the most important nonsurgical modality for the curative treatment of cancer. The success of radiotherapy depends on the radiosensitivity of the tumor cells. One of the major problems encountered in radiotherapy of cancer is the radiation damage of normal tissues around the tumour during the therapy. The methods employed to overcome this is to use radiomodifiers–hypoxic–cell sensitizers to enhance damage to tumour cells and use of radioprotectors to protect normal tissues without affecting tumour cells. The use of concurrent chemotherapy has become standard practice for a number of cancers, and the addition of new, molecularly targeted agents in combination with radiotherapy is promising to improve cure rates further (Bonner *et al*., 2006*)*. The systemic toxicities of the anticancer therapeutics negatively affect quality of life, so strategies aimed at toxicity reduction are important.

Half a century of research in search of radioprotectors have contributed practically little for human application except one compound 'amyfostin' which also has side effects and problems of toxicity in some cases. Many of the radioprotectors described in the literature are not suitable for human applications due to their toxicity and side effects (Machtay *et al.,* 2004 and

Maisin *et al.,* 1993). An ideal radioprotector should protect against acute and chronic irradiation, should be non toxic and stable under physiological conditions, should have easy availability and low cost, should have oral route of administration for clinical use in radiotherapy. Plant products have various pharmacological properties and have been used for the treatment of various diseases long ago. Therefore, screening herbal drugs offers a major focus for new drug discovery. In this way, attention over the past 15 years has shifted towards the evaluation of plant products as radioprotectors, due to their efficacy and low toxicity. Most effective studies on plants have been on total extracts for their ability to protect against radiation-induced chromosomal aberrations and micronuclei formation; they were assessed by genotoxic tests, such as micronucleus and metaphase analysis (Hosseinimehr, 2007).

The research work proposed for the thesis aims to explore some indigenous medicinal plants used in Ayurvedic system of medicine for modifying biological effects of ionizing radiation with the objective of beneficial application in human situations. The medicinal plants used for the studies are *Centella asiatica* Linn, *Rubia cordifolia* Linn and *Holarrhena antidysenterica* Wall. We have also evaluated the radioprotecting activity of the phytoceutical asiaticoside which is one of the major constituent present in the extract of the plant *Centella asiatica* L. The thesis comprises biochemical and molecular biological studies *in vitro* and *in vivo* in murine model with respect to their mechanism of action. As the main target of radiation inactivation of cells are cellular DNA and membranes, efforts are directed to monitor the damages to these vital cellular targets. The radiation induced damages to cellular DNA are monitored by alkaline single cell gel electrophoresis (comet assay), and the damages to membrane are studied by measuring peroxidation of membrane lipids. Estimation of cellular antioxidant molecules like GSH and enzymes such as GPx and SOD are also undertaken. Efforts are made to understand the anti-inflammatory activity of the extracts of the plants *Centella asiatica* and *Rubia*

cordifolia and the phytoceutical asiaticoside. We have also studied the amelioration of cisplatin induced nephrotoxicity by *Rubia Cordifolia* extract in mouse model. Further the studies were also undertaken to investigate the radioprotection by evaluating enhancement of DNA repair at sub-lethal dose and radiation induced mortality after lethal dose of gamma irradiation. Thus the present study envisages developing quick and effective screening methods for radioprotecting agents and studying their underlying mechanism and also exploring the possibility of using these agents for beneficial use in cancer radiotherapy.

Objectives

The major objective of the present study is to investigate the possibility of using medicinal plants and phytoceuticals for ameliorating the side effects of radiation in mammalian systems which will be helpful in cancer radiotherapy.

The major objectives of the present study are:

1. Preparation of the hydroalcoholic extracts of the Indian medicinal *plants Centella asiatica, Rubia cordifolia* and *Holarrhena antidysenterica.*
2. To understand the *in vitro* radioprotecting ability of the extracts and asiaticoside, the major component present in *Centella asiatica*, at the molecular level by assessing their ability to protect membrane lipids and plasmid DNA against γ-radiation induced damages.
3. To determine the ability of the extracts / phytoceutical to prevent the γ -radiation induced DNA strand breaks *in vivo* using mouse models and *ex vivo* using human peripheral blood leukocytes as measured by single cell gel electrophoresis (comet assay).
4. Evaluating the ability of the extract of *Centella asiatica* and asiaticoside to enhance the repair of radiation-induced DNA strand breaks.

5. To determine the ability of *Centella asiatica* extract and asiaticoside to prevent the radiation induced genomic instability via studying the micronuclei formation.
6. To study the effect of administration of the extracts and the phytoceutical on lethal dose of radiation induced mortality.
7. To determine the haematogical parameters and antioxidant status in asiaticoside and *Centella asistica* extract administered animals exposed to different doses of γ -radiation.
8. To study the free radical scavenging potential of the extracts of *Centella asiatica*, *Rubia cordifoila* and asiaticoside and by evaluating their anti-inflammatory activity in mouse models.
9. To assess the efficacy of the *Rubia cordifolia* extract to ameliorate the toxicity of cancer chemotherapeutic cisplatin.

Methods

Preparation of the extracts: Authenticated plant parts of *Centella asiatica* L., *Rubia cordifolia* L. and *Holarrhena antidysenterica* Wall were obtained from Amala Ayurvedic Hospital and Research Centre. The dried parts of plants were powdered and extracted (100g) twice with five times (500 mL) volume of 70% ethanol by stirring overnight at the room temperature. Extract was filtered through Whatmann No.I filter paper and the supernatant was evaporated under reduced pressure using rotary evaporator at 45^{O} C and the final liquid suspension was lyophilized to get a powder. The lyophilized powders hereafter referred as CAE (*Centella asiatica* extract), RCE (*Rubia cordifolia* extract) and HAE (*Holarrhena antidysenterica* extract). The HPTLC analysis of all the extracts were carried out using silica gel 60 F254 plates of E.MERK with the solvent system butanol:acetic acid:water (5:1:4) at the detection wavelength of 356 nm.

Determination of antioxidant status: The drug asiaticoside-50mg/kg body weight/ *Centella asiatica*-200mg/kg body weight was administered for three consecutive days and on the third day the animals were exposed to whole body γ- radiation (2, 4 or 10 Gy respective to each group). All animals were sacrificed 24 hours post irradiation. Blood was collected by heart puncture into heparinised tubes and bone marrow cells were collected by flushing the femur bones of each animal with 1ml PBS containing 10 % fetal bovine serum. The liver and brain of each animal were excised, rinsed thoroughly in ice cold PBS to remove blood. Bone marrow cellularity was determined by the method of Sredni *et al.* (1992). Total WBC count (hemocytometer method) and hemoglobin content (Drabkin and Austin, 1932) was determined. Total protein was estimated by the method of Lowry *et al*. (1951). Assay of GPx followed the method of Hafeman *et al.* (1974). Level of GSH was assayed by the method of Moron *et al.* (1979). The lipid peroxidation was measured in terms of thiobarbituric acid reacting substances (TBARS) at 532nm. The values are expressed as n moles of malondialdehyde (MDA) per mg protein (Joy and Nair, 2008).

Anti-inflammatory activity: Anti-inflammatory activity of CAE, RCE and the phytoceutical asiaticoside was determined by carrageenan induced acute and formalin induced chronic inflammation in mouse models with diclofenac as standard (Winter *et al*, 1962). Paw oedema was induced by carrageenan or formalin by subplantar injection in the hind paw of Swiss albino mice pre-administered with the extracts and asiaticoside and percent inhibition was calculated. In the case of chronic inflammation, the administration was continued for 3 consecutive days.

Determination of radioprotecting property *in vitro, ex vivo* and *in vivo*: To evaluate the effect of herbal extracts and phytoceutical on γ -radiation induced strand breaks *in vitro*, the plasmid pBR322(150 ng), in phosphate buffer (0.1M,

pH 7.4) was exposed to γ rays (0-25 Gy) in the presence and absence of the extract/phytoceutical . After irradiation, the DNA was electrophoresed, stained with ethidium bromide and the DNA bands were photographed and analyzed (Sambrook *et al*., 1989).

The effect of herbal extracts / phytoceutical on radiation induced DNA strand breaks in cellular genomic DNA were studied using single cell gel electrophoresis (comet assay) (Sing, 2000). *Ex vivo* studies were carried out using human peripheral blood leucocytes.

For *in vivo* studies, the animals administered with herbal extracts / phytoceutical were exposed to γ -radiation (4 Gy). Animals were sacrificed by cervical dislocation 1 hour after irradiation; bone marrow cells were collected by flushing the femur bones of each animal with FBS (Fetal Bovine serum) containing PBS (Phosphate Buffered Saline). Spleen was excised out and made to single cells. Comet assay was performed using bone marrow cells and spleenocytes.

To study the effect of herbal extracts / phytoceutical on 10 Gy γ -radiation induced mortality, animals were administered with the extracts or asiaticoside , 1 hour prior to whole body γ -radiation and continued for 7 consecutive days. The animals in all the groups were checked on a daily basis to record the mortalities.

Studies on enhancement of DNA repair: The efficacy of CAE and asiaticoside to enhance the repair of cellular DNA from radiation induced strand breaks was studied *ex vivo and in vivo* using comet assay. For *ex vivo* studies, mouse peripheral blood was collected into heparinised tubes. The blood samples were exposed to 4 Gy γ -radiation and incubated in the presence and absence of extract and asiaticoside. At time intervals of 0, 15, 30, 45 and 60 minutes, blood from each sample were processed for comet assay. For *in vivo* studies, animals

were exposed to 4 Gy whole body γ-irradiation and the extract / asiaticoside was orally administered immediately after irradiation. Bone marrow cells were collected by sacrificing the animals at different time intervals and were subjected to comet assay.

Studies on radiation induced micronuclei formation: The effect of CAE or asiaticoside on radiation induced micronuclei formation was studied using Swiss albino mice, exposed to 2 Gy γ-radiation. The extract/ asiaticoside was administered 1 hour prior to irradiation. Peripheral blood was collected by tail vein puncture at 24 h and 48 h after irradiation. Aliquots of the blood were kept on acridine orange coated slides and the reticulocytes and micronucleated reticulocytes (MN-RTs) were monitored using a fluorescent microscope (Hayashi *et al*., 1990).

Nephroprotective activity of *Rubia cordifolia* extract: To determine the protective effect of RCE against the side effects of cisplatin, Swiss albino mice were injected i.p. with cisplatin (12 Mg/ kg body wt). RCE was administered by oral gavage 1 h before and at 24 h and 48 h after cisplatin injection. Seventy two hours after cisplatin injection, animals were sacrificed: blood samples were collected by heart puncture for measuring serum urea and serum creatinine levels (Jisha and Nair, 2008). Kidneys were quickly removed; homogenates (10% w/v) were prepared in PBS and used for the estimation of GSH, GPx, SOD, catalase and lipid peroxidation. A portion of the kidney was fixed in 10 % formalin immediately after sacrifice and used for histopathological analysis.

Results

Radiation is used therapeutically for the treatment of various types of malignancies. It is well known that most of the damages induced by radiation to living cells are due to the generation of aqueous free radicals. Thus any compound capable of scavenging the free radicals could be used as

radioprotector. According to Umadevi (2002) antioxidant activity has prominent role in the radioprotective effects of these compounds. Oral administration of CAE could restore the decline in the anti-oxidant enzymes caused by ionizing radiation in Swiss Albino mice. Thus it was observed that the drug could enhance the antioxidant activity. In addition to cellular DNA; membranes constitute another important vital target for radiation inactivation in cells. The major radiation induced damage in membrane is peroxidation of lipids. Administration of CAE reduced radiation induced peroxidative damage to membrane lipids.

The depletion in GSH contents after exposure to gamma radiation may be due to the reaction of GSH with free radicals to produce GSSG (Navarro *et al*., 1997). In the normal conditions, the cells are intact and healthy and GSH is restored by synthesis, but in the irradiated animals the normal synthesis/repair will be disrupted due to damage to DNA and membranes. CAE treatment in irradiated mice protected GSH and the levels were close to normal. Also the hematological parameters such as, total WBC count and hemoglobin content were brought near to normal levels when the animals were administered with CAE.

During radiotherapy or chemotherapy the major target of attack is cellular DNA through generation of free radicals. Damages to cellular DNA mainly include double or single strand breaks, which can be efficiently assayed as an indicator of the extent of cell damage. Alkaline single cell gel electrophoresis is one of the efficient methods to monitor DNA strand breaks in individual cells (Wada *et al*., 2003). The analyzed results of comet assay implicated that CAE protected DNA from strand breaks significantly. Exposure to □ radiation lead to DNA strand breaks resulting in the relaxation of plasmid DNA from super coiled (ccc) form to open circle (oc) form. It was seen that the presence of the extract

reduced the radiation induced disappearance of ccc form of the plasmid DNA significantly.

The phytoceutical asiaticoside which is the major component present in *Centella asiatica*, was also found to be effective in mitigating the radiation induced damages under in *vitro, ex vivo* and *in vivo* conditions of radiation exposure.

The comet analysis of DNA of whole body irradiated mice bone marrow cells at different time intervals showed a decrease in comet parameters with the increase in time which indicates that the strand breaks in cellular DNA were repaired in a time dependent manner. When CAE and asiaticoside were administered to mice following irradiation, it was found that repair of cellular DNA damage in whole body irradiated mice is enhanced greatly. The present results also showed that the extract/ asiaticoside exhibited the ability to enhance DNA repair in human peripheral blood leucocytes irradiated under *ex vivo* conditions.

Administration of CAE / asiaticoside also protected whole body irradiated mice (2 Gy) from genotoxic effects of γ-irradiation as shown by the reduction in the number of radiation induced micronuclei formation. The administration of the extract/asiaticoside significantly reduced the frequency of micronucleated reticulocytes implying their ability to protect the mouse chromosomes from radiation induced damages.

Mortality of animals following radiation results from various syndromes depending on the doses. The whole body irradiation of mice to 10 Gy resulted in a decline in the survival of mice and oral administration of the extracts/ asiaticoside decreased radiation-induced mortality in mice. All the extracts / asiaticoside showed a decrease in the radiation induced lethality. Maximum survival advantage was exhibited by the phytoceutical asiaticoside and the least protection was obtained by HAE.

Also the studies demonstrated the potent antioxidant activity of RCE *in vivo* and *in vitro.* Our studies showed that RCE has stimulatory effect on hematopoetic system. Radioprotective activity of the extract was proved by alkaline comet assay of cellular DNA which was performed under *in vitro* and *in vivo* experimental conditions. The antioxidant activity, potent stimulation of hematopoetic system as well as non toxicity suggests that RCE has a great potential for use as a radioprotector in human studies. Administration of cisplatin to mice induced a marked renal failure, characterized by significant increase in serum urea and creatinine levels in addition to severe alterations in renal tissue structure. It has been suggested that cisplatin is able to generate ROS and that it inhibits the activities of antioxidant enzymes in renal tissues, e.g., GPx, SOD and catalase (Atessahain A *et al.*, 2004). In the present study the activities of GPx, SOD and catalase were found to be reduced in kidneys of mice treated with cisplatin. But the RCE administration restored the cisplatin induced impairments to a considerable extent. The administration of the extract also preserved normal renal tissue architecture in cisplatin intoxicated mice.

ROS and free radicals are thought to act directly as cellular messengers to elicit an inflammatory response. An anti-inflammatory drug, the activity of which is based on free radical scavenging mechanism is considered as ideal. CAE, RCE and asiaticoside were found to possess significant anti-inflammatory effects in both acute and chronic models. The anti-inflammatory activity can be attributed to their potential of scavenging free radicals from the biological system.

Summary and Conclusion

Plant products have various pharmacological properties and have been used for the treatment of various diseases long ago. The development of radioprotective agents is important for protecting patients from the side effects of radiotherapy, as well as the public from unwanted irradiation. The present study demonstrates

the use of herbal extracts for protecting the mammalian system from the deleterious effect of radiation. CAE and asiaticoside are found to be effective in protecting mice from radiation induced damages at the molecular and cellular level. Their ability to scavenge free radicals and to enhance the cellular DNA repair process may be the mechanisms underlying their radioprotection. Studies on RCE also have shown promising results. So the application of herbal extracts/ phytoceutical in human beings as an adjuvant for radiotherapy would be easy as they are either part of regular diet or constituent of a well practised herbal medicine. Although further investigations regarding the optimum dose, route of administration, a detailed pharmacokinetic profiling for human subjects are essential.

The thesis has been divided in to 8 chapters as follows:

1. Introduction & Review of literature
2. Materials & Methods
3. Studies on the plant *Centella asiatica* Linn: Protection of cellular DNA and membrane from radiation induced challenges
4. Amelioration of radiation induced damages and enhancement of DNA repair by asiaticoside.
5. Radioprotective, nephroprotective and anti-inflammatory activities of the plant *Rubia cordifolia* Linn.
6. Radiation protection by *Holarrhena antidysenterica* Wall: protection of DNA *in vitro* and *in vivo* against radiation.
7. Summary & Conclusion
8. Bibiliography

List of publications