IN VITRO AND *IN VIVO* RADIOPROTECTIVE ACTIVITIES OF *Polyalthia longifolia* AGAINST LETHAL IRRADIATION

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by

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TABLE CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST ABBREVIATIONS

ANOVA	Analysis of varians
BHT	Buthylated hydroxytoluene
DMSO	Dimethyl sulfoxide
DPPH	2, 2-diphenyl-1-picrylhydrazyl
GAE	Gallic acid equivalents
LC-MS	Liquid Chromatography-Mass Spectrometry
IC_{50}	Inhibitory Concentration at 50%
LD_{50}	Lethality Dosage at 50%
Rf	Retention Factor
HPTLC	High Performance Thin Layer Chromatography
v/v	Volume per volume
w/v	Weight per volume
TLC	Thin Layer Chromatography
SD	Standard Deviation
ABS	Absorbance
ROS	Reactive Oxygen Species
AST	Aspartate aminotransferase
ALP	Alkaline phosphate
ALT	Alanine aminotransferase
CFU	Colony Forming Units
M.I.	Mitotic Index
DRF	Dose Reduction Factor
P. longifolia	*Polyalthia longifolia*
TBIL	Total billirubin

MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
CV	Central vein
TBE	Tris-borate-EDTA
MTD	Maximum Tolerable Dose
SOD	Superoxide Dismutase
LP_X	Lipid Peroxidation
HB	Heamoglobin
WBC	White Blood Cell
RBC	Red Blood Cell
HPLC	High Performance Liquid Chromatography
MPLC	Medium Pressure Liquid Chromatography
DAD	Diode Array Detector
UV	Ultraviolet
RRLC	Rapid Resolution liquid chromatography
QTOF-MS	Accurate-Mass quadrupole time of flight mass spectrometer
ESI	Electron spray ionization
OECD	Organization for Economic Co-operation and Development

AKTIVITI RADIOPROTEKTIF OLEH *Polyalthia longifolia* TERHADAP SINARAN RADIASI MAUT SECARA *IN VITRO* DAN *IN VIVO*

ABSTRAK

Tumbuhan ubatan yang kaya dengan pelbagai bahan fitokimia dengan sifat antioksidan boleh bertindak sebagai agen pelindung sinaran radiasi maut alternatif. Kajian ini telah dijalankan untuk menilai ekstrak daun *P. longifolia* sebagai pelindung sinar radiasi maut yang berpotensi. Kuantifikasi rutin telah dilakukan untuk tujuan pemiawaian dan didapati ianya adalah 8.96 µg (0.896%) di dalam 1000 µg ekstrak daun *P. longifolia*. Kajian mikroskopi cahaya terhadap keratan melintang daun *P. longifolia* memperlihatkan kehadiran pelbagai jenis sel tumbuhan. Keputusan analisa fitokimia ekstrak menunjukkan kehadiran alkaloid, triterpenoid, tanin, saponin, antrakuinon dan glikosida. Kepekatan logam berat yang ditentukan di dalam ekstrak adalah di bawah had yang dibenarkan. Analisis HPTLC ekstrak daun metanol *P. longifolia* menunjukkan sepuluh puncak spesifik. Dua belas puncak utama dalam lingkungan 4,000 hingga 500 cm^{-1} diperhatikan dalam spektrum FTIR, yang mewakili pelbagai kumpulan berfungsi khusus. Kajian antioksidan *in vitro* menunjukkan aktiviti antioksidan yang memuaskan yang bergantung kepada dos yang digunakan berbanding dengan agen antioksidan piawai. Kandungan jumlah sebatian fenolik dan flavonoid dalam ekstrak yang diuji berkorelasi rapat dengan keupayaan antioksidan ekstrak tumbuhan yang diuji. Terapi menggunakan ekstrak *P. longifolia* menunjukkan kesan perlindungan hati terhadap perubahan biokimia dan histopatologi. Dalam ujian komet, rawatan dengan 23.88 µg/mL kepekatan ekstrak

P. longifolia selama 24 jam pada sel-sel Vero menyebabkan pengurangan kerosakan DNA sebanyak 50.94% berbanding dengan kawalan yang tidak dirawat. Dalam ujian relaksasi plasmid and komet ekstrak daun *P. longifolia* mempamerkan aktiviti perencatan yang kuat terhadap kesan kerosakan yang diakibatkan oleh H_2O_2. Peningkatan dalam nilai aberasi kromosom yang bersandarkan kepada kepekatan ekstrak juga telah diperhatikan dalam ujian *Allium cepa*. Keabnormalan yang diperhatikan adalah seperti kelekitan, c-mitosis, jambatan dan kromosom vagrant. Sel mikronukleus juga telah diperhatikan dalam interfasa. Eksperimen ini adalah laporan pertama tentang kesan perlindungan *P. longifolia* terhadap kerosakan DNA yang disebabkan oleh radikal hidroksil. Tambahan pula, dalam ujian ketoksikan oral akut, tikus betina telah dirawat dengan kepekatan ekstrak daun *P. longifolia* sebanyak 5000 mg/kg berat badan tikus dan diperhatikan untuk tanda-tanda ketoksikan selama 14 hari. Ekstrak daun *P. longifolia* tidak menunjukkan sebarang tanda-tanda kesan toksik yang menunjukkan bahawa daun tersebut tidak toksik berkaitan dengan rawatan tersebut. Seterusnya, kesan aktiviti radioprotektif *P. longifolia* telah dikaji dengan menggunakan tikus. Rawatan *P. longifolia* pada tikus menunjukkan peningkatan yang signifikan dalam jumlah hari tikus hidup (27 hari), berbanding dengan 100% kematian dalam kumpulan tikus yang didedahkan pada radiasi dalam tempoh 14 hari. Peningkatan ketara dalam kepekatan hemoglobin, sel darah merah, sel darah putih dan jumlah platelet diperhatikan pada haiwan yang menerima prarawatan ekstrak daun *P. longifolia* berbanding dengan kumpulan yang didedahkan radiasi sahaja. Pemberian ekstrak daun *P. longifolia* sebelum pendedahan radiasi juga telah meningkatkan jumlah CFU limpa dan saiz limpa relatif. Penurunan yang bergantung kepada dos rawatan dalam nilai pengoksidaan lipid diperhatikan dalam haiwan yang menerima prarawatan dengan *P. longifolia*.

Walau bagaimanapun, haiwan yang menerima prarawatan dengan *P. longifolia* mempamerkan peningkatan yang ketara dalam aktiviti superoksida dismutase dan katalase, tetapi nilai-nilai ini kekal di bawah nilai biasa di dalam hati dan usus. Prarawatan dengan *P. longifolia* sebelum pendedahan pada radiasi juga menyebabkan regenerasi semula krip mukosa dan vili usus. Tambahan pula, rawatan awal ekstrak daun *P. longifolia* juga menunjukkan kesan perlindungan ke atas kerosakan hati yang disebabkan oleh sinaran radiasi-X pada tikus dengan pemulihan struktur sel hati yang normal dan pengurangan ketara aras ALT, AST dan tahap bilirubin berbanding dengan tikus yang didedahkan dengan sinaran radiasi-X. Keputusan ini menunjukkan keupayaan radioprotektif ekstrak daun *P. longifolia* yang dimanifestasikan dalam beberapa sistem haiwan ujian. Untuk mengenal pasti sebatian antioksidan, ekstrak daun *P. longifolia* telah dikenakan fraksinasi berpandukan bioasai. Fraksi *P. longifolia* etil asetat, iaitu EtOAc_F007 menunjukkan aktiviti antioksidan tertinggi di kalangan semua fraksi yang diuji. Analisis selanjutnya dengan menggunakan kaedah LC-MS terhadap fraksi EtOAc_F007 membawa kepada pengenalan sebatian rutin sebagai agen antioksidan dalam ekstrak *P. longifolia*. Kesimpulannya, hasil kajian ini menyokong potensi penggunaan ekstrak daun *P. longifolia* sebagai produk semula jadi untuk diaplikasikan di masa hadapan sebagai pembangunan agen pelindung radiasi maut baru yang bersifat semula jadi.

IN VITRO AND *IN VIVO* RADIOPROTECTIVE ACTIVITIES OF *Polyalthia longifolia* AGAINST LETHAL IRRADIATION

ABSTRACT

Medicinal plants rich with various phytochemicals with antioxidant properties could serve as an alternative radioprotective agent. The current study was designed to evaluate the *P. longifolia* leaf extract as a potential radioprotector. Rutin quantification was performed for standardization and was found to be 8.96 µg (0.896%) in 1000 µg of *P. longifolia* leaf extract. Light microscopy of a transverse section of the leaf of *P. longifolia* revealed the presence of various plant cells. Phytochemical screening results of the extract revealed the presence of alkaloids, triterpenoids, tannins, saponin, anthraquinones, and glycosides. The concentrations of heavy metals determined in the extract were well below the permissible limit. The HPTLC analysis of the methanolic extract of *P. longifolia* leaf showed ten specific peaks. Twelve major peaks in the range of 4,000 to 500 cm-1 were observed in the FTIR spectra, which represented various specific functional groups. The *in vitro* antioxidant assays showed considerable *in vitro* antioxidant activities in a dose-dependent manner when compared to the standard antioxidant Phenolic and flavonoid content of these extracts is significantly correlated with antioxidant capacity. Therapy of *P. longifolia* showed the liver protective effect on biochemical and histopathological alterations. Moreover, histological studies also supported the biochemical finding, that is, the maximum improvement in the histoarchitecture of the liver. In the comet assay, the treatment of 23.88 µg/mL of *P. longifolia* extract for 24 h on Vero cells caused decrease in DNA damage by approximately 50.94%

compared to the unchallenged control. In the plasmid relation and comet assay, the *P. longifolia* leaf extract exhibited strong inhibitory effects against H_2O_2-mediated DNA damage. A dose-dependent increase of chromosome aberrations was also observed in the *Allium cepa* assay. The abnormalities scored were stickiness, c-mitosis, bridges, and vagrant chromosomes. Micronucleated cells were also observed at the interphase. This experiment is the first report for the protective effect of *P. longifolia* on DNA damage-induced by hydroxyl radicals. Additionally in an acute oral toxicity study, female rats were treated at 5000 mg/kg body weight of *P. longifolia* leaf extract and observed for signs of toxicity for 14 days. *P. longifolia* leaf extract did not produce any signs of toxicity which indicates that the *P. longifolia* was not toxic. Subsequently, the radioprotective effect of *P. longifolia* was studied in mice. *P. longifolia* treatment rendered remarkable improvement in mice survival (27 days), compared to 100% mortality in irradiated groups mice within 14 days. Significant increases in hemoglobin concentration, red blood cell, white blood cells and platelets counts were observed in the animals pretreated with *P. longifolia* leaf extract in comparison to the irradiation-alone group. Pre-irradiation administration of *P. longifolia* leaf extract also increased the CFU counts of spleen colony and increased spleen relative size. A dose-dependent decrease in lipid peroxidation levels was observed in animals pretreated with *P. longifolia*. However, the animals pretreated with *P. longifolia* exhibited a significant increase in superoxide dismutase and catalase activity, but the values remained below normal both in liver and intestine. Pre-irradiation administration of *P. longifolia* also resulted in regeneration of mucosal crypts and villi of intestine. Moreover, pretreatment of *P. longifolia* leaf extract also shows protective effects on X-ray irradiation-induced liver damage in mice by restoration of normal liver cell structure

and significant reduction in the elevated levels of ALT, AST and bilirubin level compared with the X-ray irradiated mice. These results suggest that the radioprotective ability of *P. longifolia* leaf extract which was manifested in several systems in experimental animal. To identify the antioxidant compound, *P. longifolia* leaf extract was subjected to bioassay-guided fractionation. *P. longifolia* ethyl acetate fraction, namely EtOAc_F007 demonstrated highest antioxidant activity among all the fractions tested. Further LC-MS analysis of EtOAc_F007 led to identification of rutin as the antioxidant agent in *P. longifolia* extract. In conclusion, the results from this study strongly imply the potential use of *P. longifolia* leaf extracts from natural product in future application for the development of natural products based radioprotection agents.

CHAPTER 1.0: GENERAL INTRODUCTION

1.1 Overview and rationale of the study

The adverse effects of radiation has begun to realize immediately after the discovery of X-ray by Roentgen (1896) and radioactivity by Becquerel (1896) and it was considered as a remarkable turning point of human health care. Basically, humans are constantly exposed to radiation either from planned included diagnostic, therapeutic uses and industrial sources or unplanned included the nuclear explosion such as atomic bomb blast, which brought tremendous damage at Hiroshima and Nagasaki, Japan in 1945 and the natural background radiation emanating from the earth or other radioactive sources (Jagetia, 2007). In general, the chances of radiation exposure have been increased extensively and this further enhance by the rapid advancement in technology which also leads to the additional radiation stresses.

Broadly, the radiation sources can be categorised into ionizing and non-ionizing radiation. Ionizing radiation can be defined as any types of electromagnetic or particles radiation with sufficient energy to knock electrons off of atoms or molecules and such phenomenon known as ionization. Ionized molecules are unstable and quickly undergo chemical changes. The amount of damage in the cell is related to the dose of radiation it receives. The types of ionizing radiation included X-ray and Gamma ray which widely used in cancer treatment. Meanwhile, non-ionizing radiation has low energy that does not directly damage in molecules. Common types of non-ionizing radiation include ultraviolet (UV) rays, visible light, infrared rays, microwaves, and radiofrequency rays (radio waves), and cell phones all emit (send out) non-ionizing radiation.

Ionizing radiation is an important modality in cancer treatment and almost 80% of cancer patients required radiotherapy during some point of their clinical

management either for curative or palliative purpose (Piya Paul *et al.*, 2011). The basic principle of radiotherapy is to destroy the cancer cells. However, the radiation also induced damage to the normal tissues which results in adverse side effects after months or years of therapy and this restricts the therapeutic doses of radiation, hence limits the effectiveness of the treatments. Consequently, when the ionizing radiation passing through living tissues it transfuses deleterious effects in biological system through direct deposition of energy into crucial bio-macromolecules or by radiolysis of water which leads to generation of reactive free radicals such as hydroxyl radicals and hydrogen radicals. Thus the overproduction of these free radicals tend to become reactive oxygen species and toxic which can interact with the critical bio-macromolecules such as DNA, proteins, or membranes and induce cell damage eventually leads to cell dysfunction and death (Hosseinimehr, 2007). In general, the amount of reactive oxygen species increases in the biological system following exposure to irradiation with sufficient dose and this directly correspond to the intensity of cell damage.

The radiation has been considered an enigma to the general public and the use of radiation for therapeutic purpose as well as spectacular advances made further increases awareness of human health and such phenomenon been always associated with some skepticism. The use of radioprotectors represents an obvious strategy to improve the therapeutic index in radiotherapy. Therefore, the development of effective radioprotectors is an area of great significance due to its wide applications in planned as well as unplanned radiation exposure to reduce the risk of radiation injury or severity of damage to normal tissues (Jagetia, 2007; Piya Paul *et al.*, 2011). Research in the development of radioprotectors world wide has focused on screening a variety of chemical and biological compounds. Among the molecular

radioprotectors, WR-2721 [S-2-(3-aminopropyl-amino)] ethyl phosphorothioic acid also known as amifostine, ethiophos (USA) or gammaphos (former USSR), is the most thoroughly investigated radioprotective drug, developed at Walter Reed Army Research Institute, under the Antiradiation Drug Development Program USA (Schuchter and Glick, 1993; Sweeney, 1979). However, the strategy becomes jeopardized when it comes to using synthetic chemical compound during radiotherapy as it associated with undesirable side effects at clinically effective doses and exorbitant cost the limitation greatly restricted in clinical use. In addition, conflicting preclinical and clinical reports formulate it convoluted to accept the use of synthetic compounds during radiotherapy in an unequivocal manner (Jena *et al.*, 2010). Therefore, the side effects profile of these compounds necessitated the search for alternative drugs, which could be less toxic and highly effective at optimum dose levels. Hence, such phenomenon diverts many researches attention towards the plants and natural products focus on new drug discovery which would be safer than available synthetic drugs.

Plant products have various pharmacological properties and have been used to treat various diseases since ancient time based on traditional medicinal system. The problem of safety of the pharmaceutical products with modern system of medicine triggers increase in global interest in medicinal plants. Therefore, medicinal plants have gained importance in the international market. Moreover, more than 50% of drugs in markets are still based on natural products. In recent years, herbal market mainly in the United States, Germany, France, India, Japan and others have become huge potential market and has great room for scientific research and technology. According to WHO (World Health Organization), more than 80% of world population relies on traditional medicines, largely plant based for their

primary health care needs. Medicinal plants are currently in demand and their popularity is increasing day by day in many parts of the world and has made a great contribution towards maintaining human health. In addition, plant extracts eliciting radioprotective efficacy contain a plethora of compounds including antioxidants, immunostimulants, and cell proliferation stimulators, antiinflammatory and antimicrobial agents, some will act in single compound as well as in combination with other compounds from the same plant. Most studies using natural plant products have focused on evaluation of radioprotective efficacy of whole extracts or polyherbal formulations, and in some cases fractionated extracts and isolated constituents (Arora *et al.,* 2005). In general, biologically active compounds isolated from plant largely contribute to medicinal field compare to the available synthetic products. This may be due to the variability in chemical structures of secondary metabolites which increasing the potential of new defense mechanisms against various radiations induce damage.

A good radioprotector should be able to protect against the deleterious effects of ionizing radiation either during therapeutic procedures as well as during nuclear accidents or background irradiation. Apart from that, the agents should meets all the prerequisites of an ideal radioprotectors including should be cheap, no cumulative or irreversible toxicity in a wide dose range, provides effective long-term protection, remains stable for a number of years without losing shelf life, and can be easily or orally administered (Arora *et al.,* 2006a; Arora *et al.,* 2006b). Apart from that, radioprotective activity of plant mediated through several mechanisms, including radicals scavenging potential, detoxifying the radiation induced reactive species, inducing cellular radioprotector such as superoxide dismutase (SOD) and glutathione, enhancing the DNA repair by triggering one or more cellular DNA

pathways and also able to delay cell division and inducting hypoxia in the tissues (Nair *et al.,* 2001). Since, plant products possess complex mixture of active chemical ingredients, therefore, it is able to contribute more on novel approaches of radioprotection and mechanistic aspects as mention above, hence, it would be safe and effective paradigm for radioprotection.

Malaysia being one of the 12 mega-biodiversity centers of the world is rich in all three levels of biodiversity, namely, species diversity, genetic diversity and habitat diversity with many plants used for medicinal and nutritional purposes. There are more than 35,000 plant species being used in various medicinal purposes around the world. In Peninsular Malaysia 1,200 species of higher plants and 2,000 species in Sabah and Sarawak are reported to have medicinal value and have been used for many generation in traditional health care system (Yoga Latha *et al.*, 2011). Therefore scientific investigation may be utilized to develop drugs for various diseases which is easily accessible, available and affordable for the poor community of bottom billion society.

Polyalthia longifolia leaf has been used as potential medicinal plants since ancient time based on traditional medicine systems. The *P. longifolia* was widely used in traditional medicine as febrifuge and tonic (The Wealth of India, 1969). Fundamentally, the selected plant should be rich in antioxidants to minimize free radical generation, a savor for macromolecules like lipids, proteins and DNA, should be able to enhance internal defense mechanism, possess properties of potential disinfectant and also a good immune rejuvenator. Hence, current study was designed to evaluate the *Polyalthia longifolia* methanolic leaf extract as a potential radioprotector.

1.1.1 Research objectives

The current study was undertaken with the following objectives:

1. To standardize the methanolic extract of *P. longifolia* leaf
2. To evaluate the *in vitro* antioxidant activity, *in vivo* hepatoprotective activity and to determine total phenolic and flavonoid contents of methanolic extract of *P. longifolia* leaf
3. To evaluate the potential genoprotective effect of the methanolic extract of *P. longifolia* leaf
4. To evaluate *in vivo* radioprotective activities of the methanolic extract of *P. longifolia* leaf using an animal model.
5. To isolate and identify of active compound/ fraction with antioxidant activity from the methanolic extract of *P. longifolia* leaf

CHAPTER 2.0: LITERATURE REVIEW

2.1 Radiation

Human is constantly exposed to lethal radiation either from planned radiation, such as during radiotherapy or unplanned radiation, such as the nuclear industry, sun's radiation and natural background radiation emanating from the earth or other radioactive sources. Once exposed to this lethal radiation, it will cause various adverse implications in our bodily system by the deposition of energy directly into the bio macromolecules, which leads to the production of free radicals, as shown in Figure 2.1. The free radicals are fundamental in modulating various biochemical processes and represent an essential part of aerobic life and metabolism (Tiwari, 2001). The most common Reactive Oxygen Species (ROS) include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-), which result from the cellular redox processes. At low or moderate concentrations, ROS exert beneficial effects on cellular response and the immune function, however, at high levels, these radicals become toxic and disrupt the antioxidant defence system of the body, which may lead to "oxidative stress" (Pham-Huy *et al.,* 2008). These reactive oxygen species, in turn, react with different bio-molecules viz., lipid, DNA, proteins and inflict oxidative damage in them (Figure 2.1).

The mediated reactions of major reactive oxygen species (ROS) include lipid peroxidation, removal of thiol group from cellular and membrane proteins, strand breaks and base alterations leading to DNA damage (Shukla and Gupta, 2010). After the widespread realization concerning the adverse effects from lethal irradiation various safety measurements were introduced to overcome this problem. However, the radioprotective system developed against the lethal irradiation, most of the time,

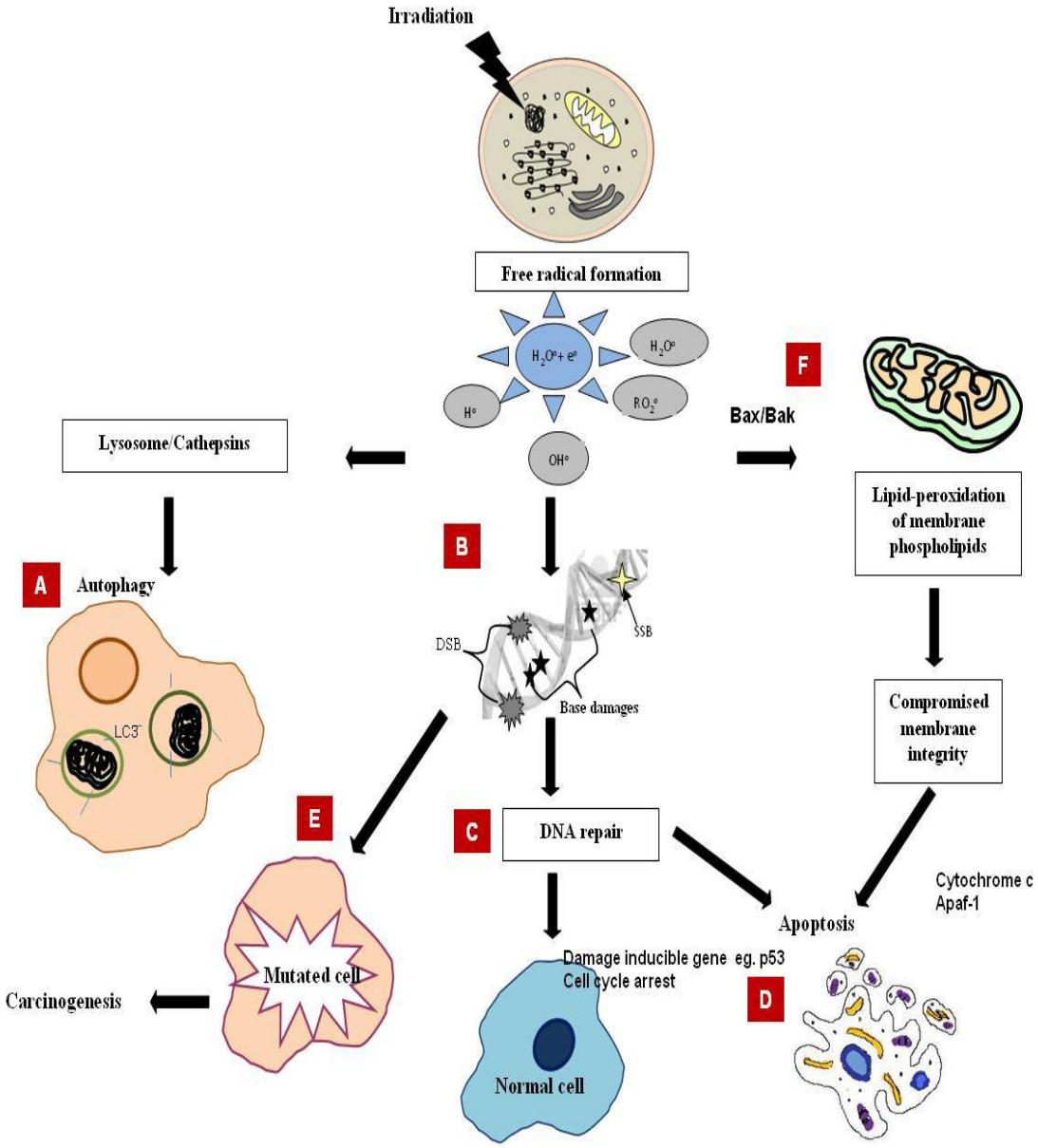

Figure 2.1: Production of free radical and related damages by lethal irradiation.

A. In response to irradiation, lysosomal proteases such as cathepsins are shuttled from the lysosomal lumen to the cytosol, resulting in autophagic cell death

B. The cell damage arises from damage to DNA due to base damages, double strand breaks (DSB) or single strand breaks (SSB).

C. In the presence of DNA damage, p53-dependent gene transcription is increased and ubiquitin-dependent degradation of the protein is blocked

D. Leading to induction of apoptosis and/or cell cycle arrest

E. Failure to activate the DNA repair mechanism in the cell leads to DNA mutation and tumourigenesis as a consequence.

F. Proapoptotic BCL-2 family members Bak and Bax sensitize the mitochondria to calcium-mediated fluxes and cytochrome c release. Cytochrome c together with Apaf-1 activates a cascade of caspases, resulting in D) apoptosis.

is burdensome to use or less practical in various situations, such as during space travel. Therefore, medicinal plants rich with various phytochemicals with antioxidant properties could serve as an alternative radioprotective agent and could be the most practical strategy to protect from lethal irradiation, which leads to various diseases including cancer. The development of safe, non-toxic, cheap, reliable and accessible radioprotective agents is crucial to overcome radiation related problems, especially for patients undergoing radiotherapy. Plants will be the ideal source to achieve this noble intention. In 1948, for the first time, Patt *et al.* (1949) discovered that cysteine (Figure 2.2) was a radioprotector agent and proved that it protected mice against the harmful effects of X irradiation. Ever since then, a number of compounds have been evaluated by various scientists from various sources including plants for the development of a radioprotective agent. Medicinal plants remain the exclusive choice for the development of a safe and effective non-toxic radioprotector since most of the medicinal plants are rich with antioxidant phytochemicals.

2.2 Ionization and formation of free radicals

When cells are exposed to radiation they interact with target atoms and deposit the energy resulting in ionization or excitation. Subsequently, the absorbed energy starts to damage the molecules directly or indirectly. The damage occurs directly through the ionization of atoms in the key molecules in the biological system, which leads to functional alteration of the molecule. Absorption of energy is enough to get rid of an electron, which results in bond breaks in the molecules. Conversely, the indirect mechanism involves ionization in the cytoplasm, which produces reactive free radicals of which the toxicity to the essential molecules results in an adverse effect and biological effects, as shown in Figure 2.1.

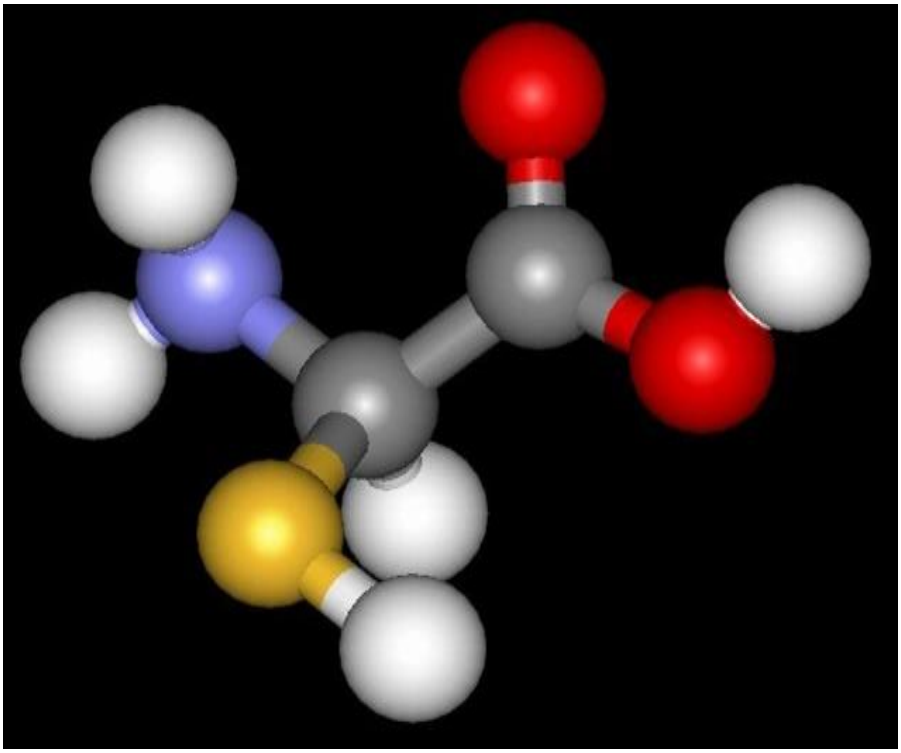

Figure 2.2: Chemical structure of Cysteine

2.2.1 Free radicals and cell death

DNA damage within the cell may occur as a result of a direct radiation hit or indirectly from free radicals (ROS). Eukaryotic cells typically respond to radiation by activating the DNA repair pathways and cell cycle checkpoints, followed by either full biological recovery or cell death (Ozben, 2007) (Figure 2.1). Radiation-induced ROS production can lead to cell death through several mechanisms including apoptosis, necrosis and autophagy (Ozben, 2007; Azad *et al.,* 2009; Wochna *et al.,* 2007).

Apoptosis is a type I programmed cell death that occurs through two main pathways, triggered either by the release of apoptotic proteins from the mitochondria (intrinsic pathway) or by death-receptor ligation (extrinsic pathway) (Edinger and Thompson, 2004). Apoptosis is depicted by membrane blebbing, early collapse of the cytoskeleton, externalisation of phosphatidylserine (PS) on the cell surface, cytoplasmic shrinkage, chromatin condensation, and, subsequently, the formation of apoptotic bodies. In contrast to apoptosis, necrosis is regarded as a passive form of cell death. Necrotic cells swell and lose their membrane integrity, then lyse and release their contents into the extracellular space, causing inflammation and damage to the surrounding tissue (Edinger and Thompson, 2004). In many cases, apoptosis and necrosis may occur sequentially or simultaneously within the same tissue due to irradiation. Through a series of well-designed studies, Wochna *et al.* (2007) hypothesised that the switch from apoptotic to necrotic cell death involves not only a diminution in cellular adenosine triphosphate (ATP) during cellular dysfunction, but also an explosion of intracellular ROS.

Mitochondria organelles are the energy powerhouse of the cell. Irradiation causes lipid peroxidation of membrane phospholipids and compromised membrane

integrity resulting in the release of small molecules including cytochrome c (Liu *et al.,* 1996) from the intermembrane space and apoptosis-inducing factor AIF (Susin *et al.,* 1999), resulting in cell death. The pro-apoptotic BCL-2 family members are mediators of cell death that reside upstream of the mitochondria (Tsujimoto, 2003). In response to irradiation, the p53 tumour suppressor induces the expression of a number of damage induced genes regulating apoptosis, including death receptors and proapoptotic members of the Bcl-2 family, Bax and Bak (Chipuk *et al.,* 2004). The p53-induced apoptosis proceeds through a series of events from the liberation of cytochrome c from the mitochondria to the activation of caspase cascades (Villunger *et al.,* 2003).

Autophagy or type II programmed cell death is caspase independent and does not involve DNA fragmentation. In autophagic cell death, organelles in the cytoplasm, including mitochondria, are sequestered in an autophagosome, which then fuses with the lysosomes (Azad *et al*., 2009). Lysosomal proteases, cathepsins, will be shuttled from the lysosomal lumen to the cytoplasm in response to ROS. The hydroxide produced, as in mitochondria by ROS, diffuses into lipofuscin-loaded lysosomes, and the hydroxide causes damage to the lysosomal membranes, which causes the leak of lysosomal enzymes. The lysosomal enzymes permeabilise the mitochondrial membranes, resulting in the release of cytochrome c, the apoptosis-inducing factor (AIF), and the second mitochondria-derived activator of caspase (Smac)/direct inhibitor of apoptosis protein binding protein with low pI (DIABLO), hence triggering cell death (Ghavami *et al.,* 2010; Szumiel, 2011).

2.2.2 Free radicals and cancer

However, the irradiated cells that escape cell death may undergo mutation, which creates an error in the DNA blueprint leading to altered gene expression and protein

modification; peptide bond cleavage and cross linking, for example, may affect protein localization, interactions and enzyme activity. Although ROS-mediated DNA damage may enable cells to function partially and proliferate, they eventually develop into cancer, especially if the regulation of the tumour suppressor genes is impaired (Wu, 2006). The high levels of ROS in cancer cells can further contribute to oxidative stress, which may further stimulate tumour growth, invasion, angiogenesis and metastasis (Wu, 2006; Girdhani *et al.,* 2005).

The level of ROS production and antioxidant signalling appear to be altered in malignant cells, contributing to cancer progression. However, the results from different studies have been paradoxical, for instance, superoxide dismutase (SOD) expression has been shown to decrease cancer cell proliferation and tumorigenicity *in vitro* (Oberley, 2005), albeit its expression was found to be associated with bad prognosis in patients with gastric cancer (Kim, 2002).

2.3 Radioprotection mechanisms by plant extract or compounds

Numerous investigations on radioprotection mechanisms have been carried out in several biological systems and the following radioprotection mechanisms have been proposed from these studies: free radical scavenger, repair by hydrogen donation to target molecules, formation of mixed sulphides, delaying of cellular division and induction of hypoxia in the tissue (Varanda and Tavares, 1998). The mechanism of free radical scavenger suggests that medicinal plants will donate electrons to the free radicals and form a stable compound incapable of reacting with other cellular components. This mechanism prevents the free radicals from reacting with the vital cell components. Another mechanism that has been proposed is the repair by hydrogen donation. If a R-H molecule is converted into a radical $R^{\bullet}$ by exposure to radiation, the antioxidant plant extract or compound can donate a hydrogen atom to

this radical, restoring it to its original state (Biaglow, 1987), which is not vulnerable to the vital components of our bodily system. In addition, the mechanism of the formation of mixed sulphides suggests aminothiols, which involves radioprotector binding to cellular components. According to this proposed mechanism, the sulphydryl compound of medicinal plants form mixed disulphides with sulphydryl compounds of cellular proteins. Once the free radicals generated by irradiation attack the disulphides, the sulphur atoms will be reduced and the other sulphur atom will be oxidized (Varanda and Tavares, 1998). This mechanism prevents the free radicals from reacting with the vital cell components because if the sulphur atom of the protein is reduced by the free radicals and the sulphur atom of the protective agent is oxidized, the protein is not damaged.

Delaying of cellular division and granting additional time for repairing DNA damage caused by irradiation has been considered a potentially important mechanism in radioprotection activity. For this type of mechanism, Brown (1967) proposed that the sulphydryl compounds of the radioprotective agents will bind to the cellular DNA and inhibit its replication and provide additional time for repair of the damaged DNA. Protection by the induction of hypoxia in the tissue has also been considered a potentially important mechanism in radioprotection activity. Oxidation of the radioprotective agents uses enough oxygen to reduce its tension, and it has already been revealed that hypoxia is radioprotective. Moreover, the induction of hypoxia in tissue in certain conditions may contribute to radioprotection. Nevertheless, other mechanisms might be involved, since some compounds exhibit radioprotective activity without altering the oxygen tension on the tissue (Varanda and Tavares, 1998). There is evidence of the existence of more than one radioprotective mechanism of a certain compound, and that one of the compounds

might be more or less important, depending on the irradiated system and on the specific radiation conditions (Prasad, 1982).

2.4 Plant as anti-radiation sources

2.4.1 Traditional usage of medicinal plant as radioprotective agent

For eons, plants and plant products have been infused in human life, as palatable and remedial sources. Traditional healers exploited plants to treat various maladies long before the discovery of drugs (Cragg *et al.,* 1997). Moreover, the conventional plant preparations are also demonstrated to be non-toxic or less-toxic, considering their derivation from natural resources.

Gingko biloba is one of the world's ancient trees and is believed to have survived an atomic bomb explosion dropped on Hiroshima on 6 August 1945 by the Americans (Anonymous, 2013a). The surviving trees were found near the blast centre and appeared to sprout without major deformations. The observation substantiates the plant's amazing resistance to mutagen agents like radiation (Pickstone, 2010). On a different occasion, the Buddhist monks took delight in tending to these trees by preserving them near the pagodas in China's Imperial Gardens and on sacred grounds to ward off fire. *G. biloba* is also denoted as a symbol of longevity.

Although folklore does not directly imply that plants impart a radioprotective effect, much evidence has been found of their incorporation in ceremonies and rituals in which specific plants are utilized. The Tulsi or *Ocimum sanctum,* for example, is worshipped along with milk, yogurt, honey and Ganga (river) water, which are consumed by the devotees at the end of the ritual (McGuire, 2012). The ancient Indian legend states that this Queen of Herbs came as an incarnation of the Hindu goddess Tulsi and is favoured by the Lord Vishnu, Krishna and Ram (Miller

and Miller, 2003). A plant with radioprotective effect can also be identified with the presence of other properties, such as anti-inflammatory, antioxidant, antimicrobial and immune modulatory (Jagetia, 2007). Likewise, Tulsi, within the confinement of Ayurveda, was used to regulate fever, relieve coughs and flu, and mobilize mucus in bronchitis and asthma. The leaves especially were used to treat tuberculosis and ringworm of the skin. The tulsi oil is rich in vitamin C, carotene, calcium and phosphorus and is also believed to possess other properties including antibacterial, antifungal and antiviral (Anonymous, 2013b).

Radiation interacts and distresses the atoms that compose the cells. The affected atoms will subsequently form free radicals that disrupt molecules, cells, tissues and organs that eventuate to the detriment of the organism (USNRC Technical Training Center, 2013). Since free radicals are responsible for inducing radiation-damage, the radioprotective property of *Panax ginseng* is associated either directly or indirectly with its free radical scavenging capability (Lee *et al.,* 2005). Ginseng refers to the root and the rhizome of *Panax ginseng* C.A. Meyer (Araliaceae), which have been conventionally utilised by the Chinese for more than 200 years. The Chinese believe that ginseng is a reservoir with a range of pharmacological roles, such as restorative, tonic, nootropic and anti-aging (Lee *et al.,* 2005).

2.4.2 Medicinal plant with radioprotective effects

Naturally occurring herbs constitute a wide variety of antioxidants, such as alpha carotene, ascorbic acids, flavones, flavanones, flavanols, stilbenoids, anthocyanins, phenolic acids, etc., which are reported to have a broad spectrum of radiation absorption properties (Bajpai *et al.,* 2005; Ashawat *et al.,* 2006; Nichols and Katiyar, 2010; Vaid and Katiyar, 2010). In addition, it has been shown that these

phytoconstituents have a synergistic photo-protective effect and can be used as sunscreen to protect cellular damage of the skin from radiation light exposure (Campos *et al.,* 2006; Afaq *et al.,* 2003). The radioprotective effect of phytochemicals has gained popularity in skin care and attention has been focused in developing topical formulations, which can be used as complementary as well as alternate medicine to heal and rejuvenate skin from various disorders (Griffiths *et al.,* 2005; Kapoor *et al.,* 2009; Saraf and Kaur, 2010; Svobova *et al.,* 2003). Some of the medicinal plants with radioprotective properties – antioxidant, anti-inflammatory and immunomodulatory – are listed in Table 2.1.

2.5 Antiradiation compounds

Antiradiation compounds are studied by *in vitro* and *in vivo* tests that assess some of these aspects. Assay of free radicals and antioxidant assay of pharmacological agents are suggested as a good means for evaluating the radioprotective potential (Jagetia, 2007). The polyphenolic compounds, especially flavonoids, ubiquitously present in plants, have been reported to possess various beneficial biological properties, most of which are attributed to antioxidant activity. It is not surprising that radioprotective potential has been reported for extracts of herbs containing flavonoids, as well as for individually isolated flavonoids. The radioprotective effect of two extracts of *Caesalpinia digyna* and the isolated compound bergenin were compared using *in vitro* methods by Singh *et al*. (2009). The *in vitro* approach compared the protective action against the damaging effect of protein carbonylation in bovine serum albumin, lipid peroxidation in liposomes, and DNA breakage in pBR322 plasmid. The study showed that the flavonoid, bergenin, from the plant is equally potent in inhibiting DNA damage as the extracts, albeit the extracts were more potent in protecting the proteins and lipids. The pBR322 model was also used in

Table 2.1: Plant with radioprotective activity or antioxidant activity

Plant species	Scientific names	component	activity	Reference
Tomato	*Solanum Lycopersicum*	Carotenoids – lycopenes	antioxidant	(Griffiths *et al.,* 2005; Saraf and Kaur, 2010; Ravichandran *et al.,* 2005)
Carrot	*Daucus carota*	β-carotene	antioxidant	(Griffiths *et al.,* 2005; Svobova *et al.,* 2003)
Papaya	*Carica papaya*	L-ascorbic acid	Antioxidant and photoprotective	(Vile , 1997)
orange	*Citrus sinensis*	L-ascorbic acid	antioxidant	(Cimino *et al.,* 2007)
Lemon	*Citrus limon*	L-ascorbic acid	antioxidant	(Apak *et al.,* 2007)
Mango	*Mangifera indica*	L-ascorbic acid	antioxidant with anti-inflammatory and immunomodulatory activities.	(Song *et al.,* 2013)
Pomegranate	*Punica granatum*	ascorbic acid	antioxidant	(Kumar *et al.,* 2009)
Celery	Apium graveolens	Flavones – 5,7,4'-	antioxidant and ROS scavenger	(Griffiths *et al.,* 2005; Svobova *et al.,* 2003)

		trihydroxystilibine		
Red clover	*Trifolium pratense*	Isoflavone – Genistein	Inhibit UV induced peroxidase production	(Widyarini *et al.,* 2001)
Soybean	*Glycine max*	Anthocyanin	Photo protective of UV radiation	(Tsoyi *et al.,* 2008)
Green tea	*Camellia sinensis*	Flavanol – Epigallocatechin gallate	antioxidant and ROS scavenger	(Katiyar *et al.,* 2000; Katiyar *et al.,* 2001; Katiyar and Elmets, 2001; Higdon, 2007; Li *et al.,* 2009; Sharangi, 2009; Kaur and Saraf, 2011b)
Milk thistle	*Silybum marianum*	Stilbenoid- Silybin,silibinin, silidianin, Silychristin	anti-inflammatory and immunomodulatory	(Katiyar, 2002; Fguyer *et al.,* 2003; Vaid and Katiyar, 2010)
Grape	*Vitis vinifera*	Stilbenoid- Resveratrol, Flavanol - proanthocyanidin	antioxidant and ROS scavenger	(Afaq *et al.,* 2003; Saraf and Kaur, 2010; Aziz *et al.,* 2005; Mantena and Katiyar, 2006)
Apple	*Malus domestica*	Flavanoid-Quercetin	antioxidant	(Erden Inal *et al.,* 2001; Korac and Khambholja, 2001)
Boldo	*Peumus boldus*	Quercetin, Flavanol-	antioxidant and anti-	(Peter *et al.,* 2006; Russo *et al.,*

		catechin; aporphine	inflammatory	2011)
Turmeric	*Curcuma longa*	Phenolic -curcumin	anti-inflammatory, antiproliferative, Photoprotective effect	(Saraf and Kaur, 2010; Garcia Bores and Avila, 2008)
Aloe vera	*Aloe barbadensis*	antraquinones	Cellular repair	(West and Zhu, 2003)
Rhubarb	*Rheum rhaponticum*	stilbene	antioxidant and ROS scavenger	(Silveira *et al.,* 2013)

assessing the protective effect of pure compounds isolated from *Phyllanthus amarus* (Londhe *et al.,* 2009). The flavonoids, quercetin 3-O-glucoside followed by rutin, offered the greatest protection on DNA as seen by the decrease in the nicked circular form of plasmid. However, the ellagitannins, namely amariin, 1-galloyl-2,3-dehydrohexahydroxydiphenyl (DHHDP)-glucose, repandusinic acid, geraniin, corilagin, phyllanthusiin D were also effective. The protective effects of these compounds on protein and lipids damage by radiation were assessed by using rat liver mitochondria. The compounds, rutin and repandusinic acid offered maximum protection against lipid damage whereas protection against carbonyl formation in proteins was highest in rutin, phyllanthusiin D, geraniin and quercetin 3-O-glucoside.

The effects of flavonoids have also been studied by using *in vivo* techniques. For example, various doses of preparation containing 12 flavonoids (FAC) from seeds of *Astragalus complanatus* protected mice from radiation damage (Qi *et al.,* 2011). Basically, FAC increased the survival rate of irradiated mice and had a protective effect on haematopoietic tissue and the immune system. The alkaline comet assay, which involves single cell electrophoresis was able to show the protective effect against DNA damage in mouse liver cells by the FAC. Studies on radioprotection have also taken advantage of the availability of synthetic drugs that have been used clinically in humans. The protective effect of troxerution, a flavonoid derivative used for treating venous disorders, was also ascertained by using the comet assay. In this study, the method assessed the protection against DNA damage in mice blood, bone marrow and tumour cells (Maurya *et al.,* 2004).

2.5.1 Modern technique for evaluation of radioprotective activity of medicinal plants

In this section various reliable methods available for the study of radioprotective activity, such as plant sample extraction techniques, *in vitro* and *in vivo* radioprotective activity were analysed and compared. Figure 2.3 shows the various steps involved in the evaluation of the medicinal plants for radioprotective activity properties.

2.5.2 Extraction

The first step in the process of screening medicinal plants for radioprotective activity is extraction. Extraction is the separation of medically active portions of plant tissue using selective solvents through various standard procedures. The extraction technique using an appropriate solvent system separates the soluble plant metabolites and leaves behind the insoluble cellular marc. The products obtained from plants are relatively complex mixtures of metabolites, in liquid or semisolid state or in dry powder form and are intended for oral or external use (Handa, 2008). The general techniques of plant extraction include maceration, percolation, digestion, hot continuous extraction (Soxhlet) and ultrasound extraction (sonication). In addition, modern extraction methods have been developed, which include microwave-assisted extraction and superficial fluid extraction. The fundamental operations of extraction include pre-washing, drying of plant materials or freeze drying, grinding to obtain a homogenous sample and often improving the analytical extraction, and also increasing the contact of the sample surface with the solvent system (Sasidharan *et al.,* 2012). The selection of a proper extraction method is the most important part of any experiment in order to obtain therapeutically potential active constituents from the plant sample. The standardization of active compounds

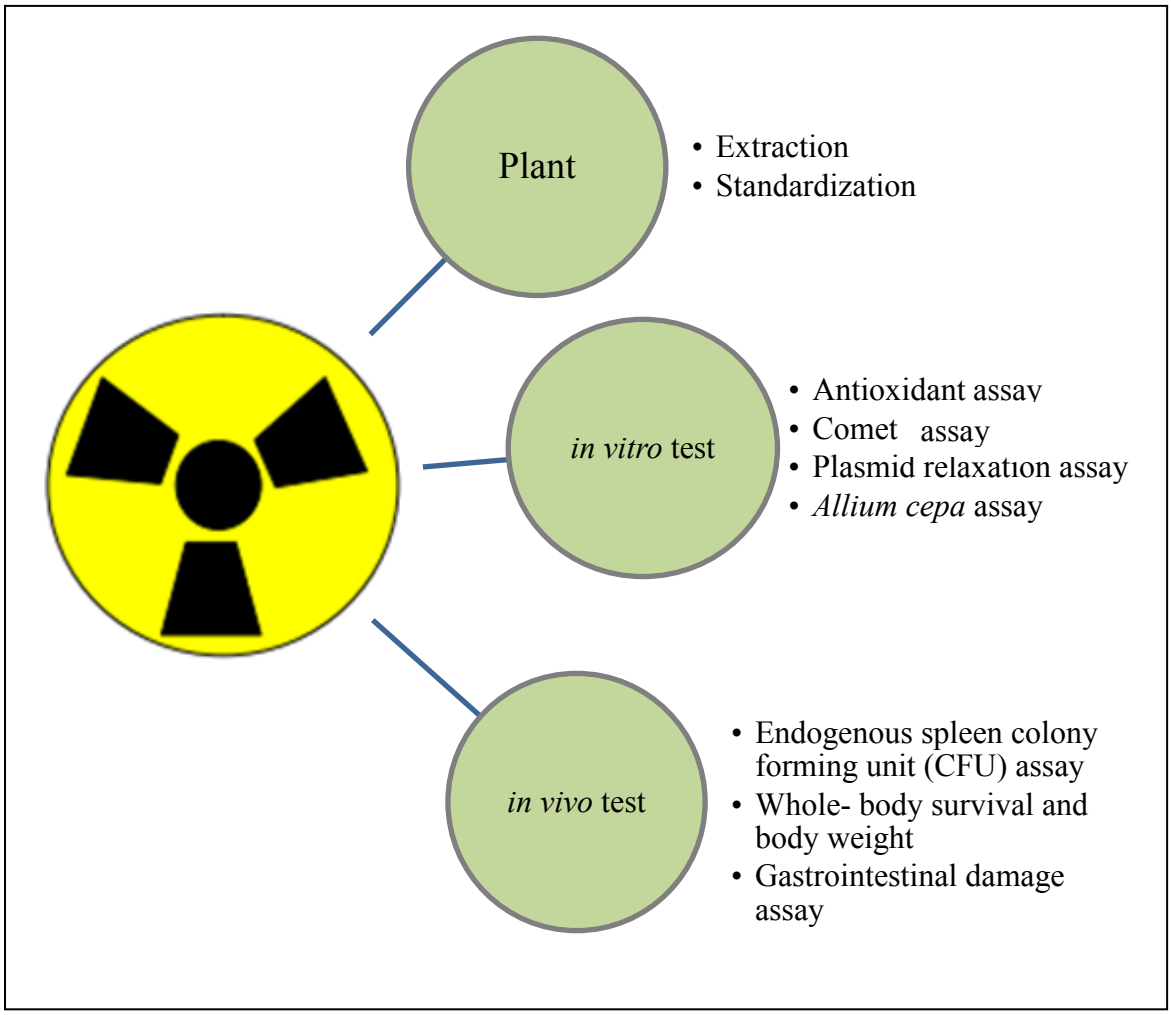

Figure 2.3: Various steps involved in the development and evaluation of radioprotective property of medicinal plants