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Review

A Novel Approach to Creating a Herbal Radioprotector Against Lethal Radiation Exposure

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Ionizing radiation transfuses deleterious effects in biological systems. Radiation exposures could be accidental or intended. Efforts for the development of a safe radioprotector have been going over many decades. Several chemical and synthetic compounds have been evaluated to meet the need, however, associated toxicity with useful doses has precluded their use from bench to bed. Presence of number of secondary metabolites, working in synergism with minimum toxicity, besides, the potential remedial source used against illness during ancient days, has compelled scientific community to exploit herbal resource for the development of a radioprotector. Published literature conveys that plants are under extensive screening for their radioprotective potentials. However, lack of a systematic approach has confined this endeavor to books and journals only. Sincere efforts have been put in the current review to explain a methodical approach for development of radioprotector from herbs. Based on thorough study, all possible steps i.e. understanding the concept of radioprotection, selection of plant species, taxonomical identification, extraction and fractionation, preservation, chemical characterization, efficacy using various model systems, toxicity and finally care to meet all regulatory requirement for development of a safe radioprotector are discussed here.

Key words: Biological radioprotection, Radiation damage, Free radicals, toxicity, antioxidants, secondary metabolites.

INTRODUCTION

The realization about adverse effects of radiation began immediately after the discovery of X-ray in the form of skin cancer. Parallely, the awareness about existence of radionucleides intensified the threat of radiation. Rapid advancement in technology also further added varied kind of radiation stresses. Broadly the chances of radiation exposure could be from planned, unplanned and natural back ground source. The planned sources are characterized as diagnostic, therapeutic and industrial sources. Unplanned includes strategic explosions, reactor accidents, fall outs, dirty bombs and terrorist attacks. Ionizing radiations inflict damage to biological systems essentially through direct deposition of energy into crucial bio-macromolecules or by radiolysis of milieu water and generation of reactive free radicals (Riley, 1994). The latter includes formation of reactive oxygen species like super oxide radicals, hydrogen peroxide, singlet oxygen and most reactive hydroxyl radicals (La Verne, 2000). These reactive oxygen species in turn react with different bio-molecules viz., lipid, DNA, proteins and inflict oxidative damage in them. Major reactive oxygen species (ROS) mediated reactions include lipid peroxidation, removal of thiol group from cellular and membrane proteins, strand breaks and base alterations leading to DNA damage. Oxidative modification of crucial functional groups in the critical membrane proteins (ion channels) and functional enzymes leads to the loss in the enzymatic activity (Beal, 2002), alterations in purine and pyrimidine bases, single and double strand breaks, removal of bases, cross linking of DNA with DNA or adjacent proteins (Scholes, 1982; Ward, 1995; Sutherland et al., 2000).

In 1948 for the first time Patt and co workers discovered cysteine as an effective radioprotector and showed that it

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can protect mice from harmful effects of total body X-irradiation when administered before radiation exposure. Since then a number of compounds have been evaluated under anti irradiation drug development program by Walter Reed. Amifostine (WR-2721), the compound from phosphorothioates group, providing good protection to haemopoietic organs against radiation, was declared the most effective compound of WR series (Weiss, 1997). During this period a large chain of chemically synthesized compounds, vitamins, amino acids, glucosides, nucleic acid derivatives etc. were tried for their radioprotective potential but could not be translated into targeted use either due to less efficacy or associated toxicity with their effective doses (Cairnie, 1983; Kligerman et al., 1984). Radiation induced multidirectional derangement can be countered holistically by administering a combination of bioactive compounds acting through different modes of action (antioxidant activity, DNA and membrane repair, regulated apoptosis, cell proliferation, immunomodulation etc.). Despite putting into unlimited man hours and input of huge finance, the available radioprotectors are not efficient for safe and sufficient inhibition of radiation energy absorption by critical macromolecules and water existing in the cell. However, last one decade has been exhaustively utilized in screening of various plants having radioprotective properties (Arora et al., 2005; Maurya et al., 2006; Jagetia, 2007; Weiss and Landauer, 2009). Considering toxicity as the major constraint with the use of synthetic compounds, numerous plants have been screened for radioprotective efficacy against the deleterious effects of ionizing radiation. Published reports are available for radioprotective properties of various plants like *Ginko bioloba* (Weiss and Landauer, 2003), *Centella asiatica* (Sharma and Jaimala, 2003), *Hipppophae rhamnoides* (Shukla et al., 2006), *Oscimum sanctum* (Devi, 2001), *Ginseng* (Song et al., 2003), *Panax* (Zhang et al., 1987), *Podophyllum hexandrum (*Mittal et al., 2001; Shukla et al., 2006; Gupta et al., 2008; Sanghmitra et al., 2009), *Tinospora cordifolia* (Goel et al., 2004), *Zingiber officinale* (Arora et al., 2005), *Allium sativum* (Jagetia and Baliga, 2003), *Hypericum perforatum* (Jagetia, 2007), *Moringa oleifera* (Katoch and Yonezawa, 1991), *Rhodiola imbricate* (Arora et al., 2005), *Morus bombysis* (Weiss and Landeuer, 2003), *Adhatoda vasica* (Kumar et al., 2003), *Hericium Erinaceus* (Benzie and Strain, 1996) and *Piper betel* (Bhattarcharya et al., 2005).

Hundreds of publications have emerged during last one decade indicating radioprotective properties of various plants. However, nothing has yet come out in the product form for human application against likely radiation exposures. Current review explains various steps starting from selection of plant material, its identification, extraction, fractionation, preservation, chemical characterization, check on radioprotective efficacy using in vitro, in vivo and ex vivo model systems, toxicity, quality control and regulatory requirement essential for development of a potential and minimally toxic radioprotector from herbs for human applications.

Expected potentials of an ideal radioprotector

The ideal radioprotector should have the following characteristics:

a) Efficient in providing multifaceted protection against undesired effects of radiation.

b) No/minimal adverse effects on the majority of organs.

c) Preferable route of administration either oral or intramuscular.

d) It should reflect an effective time-window and acceptable stability profile.

e) Compatible with the wide range of other drugs that will be made available during clinical care.

f) The formulation should have sufficiently long shelf life, easily accessible and economically viable.

g) Lastly a radioprotector for emergency need should be effective in minimum time period and efficacy should be maintained for longer durations. The formulation has to reach to all the organs and also to be able to cross blood brain barrier.

Systematic approach for development of radioprotector from herbal source

Thorough search and a clear understanding are essential before selecting any herbal source for achieving desired radioprotection. 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. Presence of rich quantity of essential minerals and vitamins also support speedy recovery in a radiation exposed person. The selected herb should have bona fide reasons to combat radiation, for instance, publications on high altitude plants like *Podophyllum hexandrum* and *Hippophae rhamnoides* indicate that their high altitude habitat has been the key criteria for selection. The logic used in favor of this statement reflects that existence in severe and adverse climatic condition probably generated anti-stress molecules as adaptogens in them and these molecules have supported these plants in combating divulged climates.

Taxonomical authentication of selected plant

Selection of a particular genus and species of the plant has to be verified by a qualified ethno botanist. Geographical distribution, age and specific part (leaf, flower, fruit, root, seed) of the plant with an indication of

whether fresh, dried or traditionally prepared material used should be clearly spelled. Disclosure regarding suitable period for collection is crucially important for getting repeatability of the observations. Voucher specimen, representing each batch of selected material with a proper label of date and batch number should be deposited in well recognized repository for at least a period of 10 years.

quality control

Stringent quality control is mandatory for herbal materials. Assays to rule out any microbial and bacterial contaminations are to be conducted. Analytical tests also should be performed to consider safety norms for heavy metals and pesticides. Numerous other parameters like dry mass, total ash and solvent extract values also need to be addressed. Plant extracts should be fully characterized including their impurities. To avoid batch to batch variation the raw should be collected from same geographical location with matched climatic conditions. Age of the plant should also be important criteria while collecting the material. Every fresh batch should pass through HPLC and HPTLC screening to ascertain the repeatability of active principles. Storage conditions such as temperature, light sensitivity, moisture etc. for both raw and fractionated material have to be clearly defined. Active shelf life of extract has to be checked using chemical and biological end points.

Extraction, Fractionation and Characterization

Methods for plant material preparations including powdered forms, extracts, essential oils etc are to be explained in details. If dried, it is essential to include the drying temperature and humidity. The specifications of finished product are also to be clearly defined.

Plant materials are fractionated using solvent of varying polarity (non polar to polar). Fractions obtained are designated based on the used solvent. Codes to different fractions are to be assigned to prevent any biasness in the application process (Markham, 1989). 'Chromatographic fingerprinting' is essential to ensure consistent quality of product. If possible, phytochemical screening has to be performed using latest techniques like LC/MS, LC/MS/MS and NMR. Efforts are to be put to identify the active principles in finished product. Their structure formulae should be qualitatively authenticated using commercially available standards. Various published reports are available as a support for these studies (Gupta et al., 2007).

Bioactivity Check

Finished products with all the valid details are to be processed further for their various bioactivity credentials using various experimental model systems. While making all the efforts it is essential to remember that humans are the ultimate user and also the product should be acceptable to FDA.

Essential assays for analysis of radioprotective qualities *IN VITRO* screening

When cells are irradiated, damage is produced primarily by free radicals which are neutral atoms or molecules having an unpaired electron. Their life span is tiny and can damage cellular molecules such as DNA, RNA and proteins within nano seconds. A potent radioprotector has the capacity to scavenge free radicals formed during irradiation and inhibit the chain propagation steps of free radicals. There are several free radical scavenging assay, some commonly used are described below.

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity

Free radical scavenging ability is based on reduction of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) to diphenylpicrylhydrazine. Mixing of DPPH with a plant extract that can donate a hydrogen atom results into the formation of diphenylpicrylhydrazine with the loss of violet colour. The decrease in absorbance is measured at 517 nm against methanol (Shimada et al., 1992).

Hydroxyl radical scavenging

2-deoxy ribose (2-DR) degradation is a simple and reliable technique to assess the scavenging ability of any agent. The 2-deoxy ribose generates hydroxyl radicals after radiation treatments which are known to induce DNA damage. Inhibition of radiation mediated break down of 2-DR is measured at 512nm (Gutteridge, 1988).

Superoxide anion scavenging

Superoxide anion is another species of free radicals that has been implicated in several pathophysiological processes, specifically due to its transformation into more reactive species such as hydroxyl radical which leads lipid per-oxidation. Numerous reports have explained antioxidant properties of herbal compounds via scavenging of superoxide anion radical. The decrease in the reduction of nitroblue tetrazolium by superoxides anions is measured at 560 nm (Kakkar et al., 1984).

Plasmid relaxation assay

The induction of single-strand or double-strand breaks in plasmid DNA results in the conversion of fast-migrating

super coiled DNA into the more slowly migrating open circular form and a linear form with intermediate migration. Evaluating these changes in plasmid is a sensitive method for assessing DNA damage. The plasmid assay has been reported to study the pro-oxidant and antioxidant properties of various compounds (Shukla et al., 2006).

Metal chelation activity

Iron is an essential metal for numerous cellular functions such as DNA synthesis, cellular respiration etc. However, when iron is in excess and unbound to protein molecules like transferrin and ferritin; it can be toxic to vital organs. Radiation is known to induce release of iron from its storage sites and this free iron amplifies the deleterious effects of radiation. Metal chelation ability of various herbal compounds/extracts is reported enormously (Benzie and Strain, 1996). The metal chelation is measured at 532nm and expressed as percentage inhibition of iron–2, 2-bipyridyl complex (chromogen) formation.

Nitric oxide scavenging activity

Radiation also generates reactive nitrogen species which are reported to act in modulation of immune response. Evaluation of nitric oxide using Griess reagent is a commonly used method to access the nitric oxide scavenging activity of a test compound (Subhalakashmi and Bansari, 2006).

Reducing potential

The antioxidant activity of an agent is known to have a direct correlation with their power to act as reducing agent. The reducing potential is evaluated as the ability to reduce ferric ions to ferrous leading to the formation of a colored complex with 2, 2-bipiridyl which is measured at 532nm (Yen and Duh, 1993).

Total Antioxidant capacity

The total antioxidant power of any radioprotectant is estimated spectrophotometrically by quantifying the formed amount of phosphomolybdenum complex (Prieto et al., 1999).

Lipid peroxidation

Radiation produces hydroxyl, superoxide and hydrogen peroxide radicals. These radicals attack cellular lipid molecules and lead to the formation of chains of free radicals. The products of peroxidation are measured as lipid hydroperoxides (LOOH), thiobarbituric acid reactive substances and 4-hydroxynonnenal (Beuge and Aust, 1978; Yang et al., 2003).

Protein oxidation

Reduction in carbonyl content by herbal compound gives preliminary idea about its protective nature against radiation mediated protein damage. Carbonyl content is measured spectrophotometrically (Staldtman, 2006).

Comet assay

Ostling and Johnson, 1987 presented the novel idea of using gel electrophoresis performed on individual cells to measure initial levels of DNA damage using this technique. Any single cell can be analysed for DNA damage including cells from invertebrate and plants. When the cell number is limited this is the most practical method to analyze damage in DNA. The damage measurement can be done in *vitro* and *in vivo.* Any diminution in DNA damage by any radioprotective compound is calculated in the form of percentage of head to tail ratio of a comet (Gupta et al., 2008).

Micronuclei estimation

Micronuclei estimation has been among one of the acceptable cytogenetic end point used in genotoxicity experiments. Micronucleus is fragment chromosome or a whole chromosome that lags behind at anaphase during nuclear division. Radiation is known to induce micronuclei in cells which give the direct evidence of chromosomal damage. Currently this assay is in use as a biomarker for evaluation of approximate radiation absorbed dose. Decrease in micronuclei formation in cells has been shown by numerous compounds against radiation (Fenech, 2000).

Measurement of endogenous antioxidant enzymes

Antioxidant defense enzymes present in the cells regulate the homeostasis balance. However, radiation exposure leads to disturbance in the defense system which results into upregulation/down regulation of the antioxidant enzymes. Glutathione S transferase, catalase and superoxide dismutase are commonly studied for evaluation of endogenous antioxidant enzymes activity. Published reports have shown the modulation of enzymatic activity in vitro and in vivo by various plant extracts (Mittal et al., 2001). Reduced glutathione which helps cells to protect from reactive oxygen species such as free radicals and peroxides could also be measured to evaluate the effect of compounds on endogenous antioxidant molecules.

Cell cycle analysis

Cells exposed to ionizing radiation become temporarily arrested at various stages in the cell cycle. The extent and the duration of the cell cycle block depend on the dose of radiation and the stage at which cells are irradiated. Herbal compounds have shown the ability to increase the population of S phase cells indicating proliferation stimulation. Radiation induced G2M delay has been exhibited by compounds which explains the allowed time for DNA repair and reduces the impact of DNA damage (Hofer et al., 1993).

Molecular studies

The mechanism of radioprotection could be evaluated based on the end point of various observations. Cell death, DNA damage repair, cell cycle proteins and biochemical measurements etc. have been commonly used by investigators to study radioprotective mode of action. Role of different signaling molecules and various signal transduction pathways are also found essential for deciphering radioprotective mechanisms (Harvey et al., 2008; Mary and Sylvain, 2006).

IN VIVO **screening assays**

The drug development process is a time consuming activity and needs a systematic and focused approach to achieve the goal. The preliminary in vitro assays results provide an idea about basic properties of the candidate agent. However, it has already been validated that obtained *in vitro* data are often not corroborated with in vivo observations. This makes mandatory for a researcher to perform experiments in laboratory animals. The commonly used *in vivo* assays are mentioned here.

Estimation of maximum tolerable dose (MTD) and Toxic doses (LD50, LD100)

The concentration of drug which does not bring about any death/toxic manifestations in the experimental animals is considered as MTD. The doses causing 50% and 100% mortality are defined as LD50 and LD 100 respectively (Gupta et al., 2008).

Whole body survival, Body weight and Dose modifying factor

Radioprotective efficacy of compounds/fractions are evaluated in the animal model by their pre irradiation administration via different routes like intraperitoneal, intramuscular, intravenous and oral followed by whole body irradiation. The effective time interval between administration of radioprotective drug and irradiation is also evaluated using survival assay. Formulations are usually studied in different concentration to find out the most effective range. Survival is ideally observed daily up to 30^{th} post-irradiation day, and the data is expressed as % survival. Animals used for this study are distributed into different treatment groups, including normal control, irradiated control, drug control, and drug plus radiation. The body weight of the animals is recorded every alternate day. The change in the average weights of the animals at different time intervals is calculated in consideration of the initial body weight of animals. The dose modification factor (DMF) can be calculated against supra lethal doses of radiation in presence or absence of radioprotective formulation (Gupta et al., 2008; Sanghmitra et al., 2009; Lata et al., 2009).

Spleen colony forming unit (CFU) Assay

Radiation affects the colony forming ability of undifferentiated cells which are assumed to be stem cells having capacity to form colony. To study this parameter, mice are exposed to varied doses of irradiation, thereafter, injected with spleenic cells of normal mice. The spleen of irradiated animals following 10^{th} post treatment day shows the discrete nodules on spleen which can be stained and counted. The effect of various doses of radiation on endogenous CFU and its modulation by administration of herbal formulations is enormously reported (Gupta et al., 2008).

Haematological assays

Total leucocytes (TLC), differential leukocytes (DLC), erythrocytes counts and haemoglobin content (Hb) measurement are routinely used parameters for studying hematopoietic effects of radiation. Total leucocytes counts get severely affected on radiation exposure. Fall in these counts start immediately after exposure to high doses of radiation. Depression in blood cell erythrocytes count and Hb content is observed only after a week of irradiation. Blood cell count, using whole blood, can be performed at regular intervals either manually or on hematology counters. Modifying effect of any radioprotective formulation can be comfortable analyzed observing augmentation in total blood cell counts (Gupta et al., 2008; Sanghmitra et al., 2009).

Gastrointestinal damage assays

Death in irradiated mice following exposure of high doses of radiation has been repeatedly reported due to severe intestinal damage. Diarrohea, loss in body weight, impaired absorption, declined electrolytes and microbial infections due to damage in intestinal layers are the most commonly observed symptoms in radiation mediated GI

syndromes. Ulcers in the lower ileum and caecum of intestine are also of general occurrence after radiation exposure. Herbal drugs have shown the ability to protect against radiation induced intestinal damages. Histological observations are most widely used method for these studies (Bertho et al., 2008; Vigneulle et al., 2002).

Central nervous system damage assays

It is assumed that adult nervous tissue is highly radioresistant however, functional changes in the neurons occur at comparatively low doses of radiation. The development of radiation-induced changes in the brain during and after irradiation depends upon several biophysical factors. Behavioral studies are also reported as equally important to study the effects of radiation and radio-protectants (Wong et al., 2004).

Pharmacokinetics and pharmacodynamics

The understanding of absorption, distribution kinetics, metabolism and excretion of any externally administered substance is an essential requirement for understanding the efficacy and toxicity of the formulation. This study is only possible when herbal preparation are processed and refined from whole extract to active principles.

Requirement of animal model systems

Animal models, being closure to the ultimate user, provide wealth of information for finalising any product for later use. Experiments in the animal models are essentially unavoidable due numerous reasons including efficacy, toxicity, dose selection, effective duration, understanding mode of action etc. Moreover, experimentation directly on ultimate user is against all ethics. In vivo models are especially important in proof-of-principle experiments and in establishing the preclinical safety and efficacy data required for progressing to human clinical trials. Mice, being small, easy to handle and broadly close to human system, are most extensively used animal model for radioprotection studies. However, the fine difference in physiology and biology among mice and human makes it imperative to use higher mammals like primates for few final experiments.

Toxicity

Herbal product has always invited controversies in accepting their non-toxic status

Almost 60-70% of world population use herbal medicines as their primary health care. Assessment of safety and efficacy of these medicines is an important issue for health professionals. In ascertaining whether the substance is associated with an adverse effect, the available literature may not be very supportive. Hence it becomes imperative to go for detailed toxicity check irrespective to whether source is herbal or synthetic. Crude herbal preparations may have extrinsic or intrinsic effects. Certain factors such as misidentification, lack of standardization, contamination, adulteration, and incorrect dosage can further multiply their toxicity. Toxicity studies generally involve sub-chronic, chronic and acute effects of any compound either to be used single or in combination (Frank and Kenneth, 1985). Toxicity studies must reflect all the data indicating any adverse effect on potential organs such as liver, kidney, lungs, spleen, bone marrow, thymus, immune system and hematopoietic organs. The key parameters to be included are biochemical,haematological, histopathological and molecular. Toxic and effective doses should be efficiently apart from each other. Life savings drugs enjoy marginally liberal norms for toxicity tests. 'Herbs are never harmful' is simply a myth. They are our natural treasures with immense potential, provided we exploit them in scientifically proven conditions.

Regulatory requirements

Development of any drug for human use has to follow stringent regulatory rules. The herbal formulations in chemically unidentified forms are not accepted as drug by Food and Drug Administration (FDA) of various countries. In semi-purified forms they are permitted to be used either as nutraceuticals or health supplements. As per FDA, USA rule (Federal Register, 2002) for radiological or nuclear substances, where well-controlled clinical studies in humans cannot be ethically conducted, certain new drug developed for preventing life-threatening conditions may be approved based on evidence of effectiveness derived from appropriate studies in animals. Simultaneously efficacy studies have to be shown in well understood animal models and substantiated in multiple appropriate species. Human clinical data on safety, toxicity, and immunogenicity is must for approval. Characterization data, mechanism of action, in vitro study, activity in condition of similar pathophysiology, pharmacokinetics, pharmacodynamics, interactions, synergy or antagonism with medical products likely to be used concomitantly or in combination are essentially needed for substance intended to use against radiological or nuclear agents. Similarly efficacy data on end points, timing of intervention, route of administration and dosing regimen are must for any new drugs to obtain FDA clearance. The regulatory body of developing countries like India is yet to come up with guidelines against radiological or nuclear agents.

CONCLUSION

Radiation induced multi organ failure is the potent cause for loss of life. Effects, being non- specific, have made all the efforts futile in development of a safe and efficient radioprotector against lethal irradiation. Initially numerous chemical compounds were screened for their radioprotective efficacy, however, due to undesired toxicity their use could not be justified. The development/evaluation of an agent which could be minimally toxic and effective against high doses of radiation is posing a great challenge to researchers. The idea of selecting plants to develop a radioprotector comes from the very fact that plants are known to act in holistic manner with comparable lower toxicity, besides, their extensive use in ancient systems of medicine like Ayurveda make them a promising alternative of chemical agents. The development of radioprotector demands multidisciplinary expertise. It is atypical to note that large numbers of plants have been reported for their radioprotective properties however, nothing has come out so far for human application. Lack of streamlined approach seems to be the key reason behind this failure. Ideally if a plant preparation shows in *vitro* and *in vivo* efficacy against radiation then in-depth exploration should be initiated instead of looking for further screening of more flora. Insertion of high through put assays will be of tremendous help in understanding the complex signaling pathways involved in the radioprotection. Understanding about the mechanism of action of the active principles, knowledge about their chemical nature, sufficiently large shelf life, easy to administer, wide absorption and distribution are some of the prime requisites for clinical trials. Toxicity studies are most important and should be done in elaborate manner at a credited laboratory. Quality control, fractionation and characterization steps serve the basis of further studies hence need careful observation. The currently available radioprotective compounds are not generally multifaceted in their action and to save biological system against lethal irradiation no single mechanism will suffice the need in rendering required protection. Authors sincerely feel that a definite and systematic path towards the development of herbal radioprotector will certainly deliver a highly potential and safe drug for human use.

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