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Triphala, an Ayurvedic *Rasayana* Drug, Protects Mice Against Radiation-Induced Lethality by Free-Radical Scavenging

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ABSTRACT

The effects of 10 mg/kg of triphala extract (TE) was studied on radiation-induced sickness and mortality in mice exposed to 7–12 Gray (Gy) of γ-irradiation. Treatment of mice with triphala once daily for 5 consecutive days before irradiation delayed the onset of mortality and reduced the symptoms of radiation sickness when compared with the non-drug double distilled water treated irradiated controls (DDW). Triphala provided protection against both gastrointestinal and hemopoetic death. However, animals of both the TE + irradiation and DDW + irradiation groups did not survive up to 30 days post-irradiation beyond 11 Gy irradiation. The $LD_{50/30}$ was found to be 8.6 Gy for the DDW + irradiation group and 9.9 Gy for TE + irradiation group. The administration of triphala resulted in an increase in the radiation tolerance by 1.4 Gy, and the dose reduction factor was found to be 1.15. To understand the mechanism of action of triphala, the free radical scavenging activity of the drug was evaluated. Triphala was found to scavenge $^{\bullet}OH$, $O_2^{\bullet-}$ 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) diammonium salt $(ABTS)^{\bullet+}$ and $NO^{\bullet}$ radicals in a dose dependent manner.

INTRODUCTION

Radiation is an important modality in the treatment of cancer and in some instances it may be the single best agent for treatment. However, a major problem associated with cancer radiotherapy is the severe side effects resulting from normal tissue damage. Consequently, agents which protect normal tissues against the radiation-induced damage can increase the patient's tolerance to radiotherapy and ameliorate radiation sickness. Historically, the sulphydryl compounds were among the first radioprotectors to be identified. Patt et al. (1949) reported that the natural amino acid cysteine protected rats and mice against radiation-induced sickness and mortality. Since then, several compounds with varied chemical structures and pharmacologic properties have been screened for their radioprotective ability in mammals. However, these compounds appear to produce serious side effects and are considered to be toxic at the doses required for radioprotection (Sweeney, 1979). The herbal drugs offer an alternative to synthetic compounds and have been considered either non-toxic or less toxic, and this has given impetus to screen for their radioprotective ability. The mechanism of action of herbal drugs and their preparations differ in many respects from that of the synthetic drugs or single substances; it can be characterized as a polyvalent action and interpreted as additive or, in some cases, potentiating (Wagner 1988).

Ayurveda (in Sanskrit, *Ayu* = life and *veda* = knowledge), the Indian system of medicine dating back 5000 years, has been an integral part of Indian culture and materia medica. According to the Ayurvedic system of medicine, the body is composed of *tridosha vata*, which is a combination of the two elements space and air and is concerned with inducing activity of the nervous system; the *pitta* consists of fire and water; and *kapha* is composed of water and earth. Radiation disrupts all the three humors and hence any drug that can restore the balance can ameliorate the damage.

Triphala is one of the important *rasayana* drugs, used since time immemorial and described in the Ayurveda as a "tridoshic rasayana" (Charka 1500 BC), having balancing

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and rejuvenating effects on the three constitutional elements that govern human life (*vata*, *pitta*, and *kapha* (Sharma and Dash, 1998). In Ayurveda, *rasayana* is a term for a therapy that produces sturdiness of the body, the sense organs, and the teeth, prevents wrinkles, graying hair, and promotes the immune function intellect, and longevity (Sharma and Dash, 1998). It is credited with diverse beneficial properties such as anti-aging, antimutagenic, anticancer, antibacterial, antiviral, cardioprotective, hepatoprotective, antistress, cleanser of colon, gas distentioner, antidiabetic, antiparasitic, antidiarrheal and antianemic (Ahmad et al., 1998; Mehta et al., 1993; Nadkarni, 1976; Niwa and Fu, 1995; Phadke and Kulkarni, 1989; Valsaraj et al., 1997). The earlier study has shown that triphala possesses protective action against the deleterious effects of radiation (Jagetia et al., 2002). However, the detailed study on the radioprotective action of triphala and its mechanism of action is lacking. The dose reduction factor (DRF) is an important aspect as it clearly gives the drug's quantitative capacity to enhance the tolerance of tissues to radiation. Therefore, the present study was carried out to obtain an insight into the radioprotective effect and mechanism of radioprotection by triphala in mice whole-body-exposed to different doses of γ-radiation.

MATERIALS AND METHODS

Animal care

The animal care and handling was done according to the guidelines set by the World Health Organization, Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India). Male Swiss albino mice, 8–10 weeks old, weighing 30–36 g, were selected from an inbred colony maintained under controlled temperature (23 ± 2°C), humidity (50 ± 5%), and light (14 h and 10 h of light and dark, respectively). The animals were provided with sterile food and water *ad libitum*. Four animals were housed in a polypropylene cage containing sterile paddy husk (rice hulls), procured locally, as bedding, throughout the experiment. The study was cleared by the Animal Ethical Committee of the Kasturba Medical College, Manipal, India.

Chemicals

Dimethyl sulphoxide (DMSO), deoxyribose, ethylene diamine trichloroacetic acid (EDTA), ascorbic acid, nitoblue tetrazolium, sodium nitroprusside, Greiss reagent, and 2,2′-azinobis (3-ethyl benzothiazoline-6-sulphonate) diammonium salt (ABTS), were procured from Sigma Chemical (St. Louis, MO). Ferric chloride, sodium bicarbonate, sodium chloride, potassium hydrogen phosphate, disodium hydrogen phosphate, potassium chloride, and hydrogen peroxide were procured from Ranbaxy Fine Chemicals (New Delhi, India).

Composition of the drug

Triphala (*tri* = three, *phala* = fruits) is a mixture of fruits of three plants: *Terminalia chebula Retz.* (Family Combretaceae), *Terminalia bellerica* (Gaertn.) Roxb. (Family Combretaceae), and *Phyllanthus emblica* (Linn) or *Emblica officinalis* (Gaertn.) (Euphorbiaceae) in powdered form in equal proportions (1:1:1).

Preparation of the extract

The aqueous extract of triphala powder was prepared as described in the Ayurvedic text and as described elsewhere (Jagetia et al., 2002).

Preparation of the drug and mode of administration

The required amount of triphala extract (TE) was dissolved in sterile double distilled water (DDW). The DDW or drug was intraperitoneally administered once daily for 5 consecutive days before irradiation.

EXPERIMENT 1. RADIOPROTECTIVE ACTIVITY

A separate experiment was carried out to ascertain the radioprotective activity of TE, with the animals divided into the following groups:

DDW + irradiation group

The animals of this group were administered 0.01 mL/g of sterile DDW intraperitoneally.

TE + irradiation group

The animals of this group were injected intraperitoneally with 10 mg/kg of TE consecutively for 5 days (Jagetia et al., 2002).

Irradiation

One hour after the last administration of DDW or TE on the day 5, the prostrate and immobilized animals (achieved by inserting cotton plugs in the restrainer) of both groups were whole-body exposed to 7 to 12 Gy of ^{60}Co gamma radiation (Gammatron, Siemens, Germany) in a specially designed well-ventilated acrylic box at a dose rate of 1.33 Gy/min at a source to animal distance (midpoint) of 102 cm. Immediately after irradiation, the animals were sorted into individual polypropylene cages. The animals of both groups were monitored daily for the symptoms of radiation sickness, and mortality up to 30 days post-irradiation. A minimum of 12 animals were used for each dose of radiation for each group and a total of 288 animals were used for this experiment. The DRF was calculated by the method of Miller and Tainter (1944):

$$\text{DRF} = \frac{\text{LD}_{50/30} \text{ of TE + irradiation}}{\text{LD}_{50/30} \text{ of DDW + irradiation}}$$

EXPERIMENT 2. FREE RADICAL SCAVENGING *IN VITRO*

A separate experiment was carried out to understand the mechanism of action of TE.

Hydroxyl radical scavenging activity

The scavenging of hydroxyl ($^{\bullet}$OH) free radicals was measured by the method described by Halliwell et al., (1987). Briefly, the reaction mixture contained deoxyribose (2.8 μmol), KH_2PO_4 −NaOH buffer, pH 7.4 (0.05 μmol), $FeCl_3$ (0.1 μmol), EDTA (0.1 μmol), H_2O_2 (1 μmol), ascorbate (0.1 μmol), and TE (10–500 μg/mL) in a final volume of 2 mL. The reaction mixture was incubated for 30 minutes at ambient temperature followed by the addition of 2 mL of trichloroacetic acid (2.8% w/v) and thiobarbituric acid. The reaction mixture was kept in a boiling water bath for 30 minutes; after cooling the absorbance was read at 532 nm in a UV-VIS double beam spectrophotometer (UV-260, Shimadzu Corp., Japan).

Superoxide anion scavenging activity

The scavenging of superoxide ($O_2^{\bullet -}$) anion was measured as described by Hyland et al., (1983). Briefly, the reaction mixture contained varying concentrations of TE (10–500 μg/mL), nitroblue tetrazolium, and alkaline DMSO. The blank consisted of pure DMSO instead of alkaline DMSO. The absorbance was read at 560 nm in a UV-VIS double beam spectrophotometer.

Total antioxidant activity assay

Total antioxidant potential was determined by (ABTS) 2,2-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt) assay described by Miller et al. (1996). This technique measures the relative ability of antioxidant substances to scavenge the $ABTS^{\bullet +}$ radical cation generated in the aqueous phase. The reaction mixture contained ABTS (0.00017 mol), TE (10–500 μg/mL), and buffer in a total volume of 3.5 mL. The absorbance was measured at 734 nm, using a UV-VIS double beam spectrophotometer.

Nitric oxide scavenging activity

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction as described previously. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide (Marcocci et al., 1994), which interacts with oxygen to produce nitrite ions that can be estimated by use of Greiss reagent (1% sulphanilamide, 2% H_3PO_4, and 0.1% napthylethylenediamine dihydrochloride). Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitric oxide. Sodium nitroprusside (5 μmol) in phosphate-buffered saline was mixed with different concentrations of TE (20–400 μg/mL) and incubated at 25°C for 150 minutes. The samples were made to react with Greiss reagent. The absorbance of the chromaphore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was read at 546 nm and referred to the absorbance of standard solutions of potassium nitrite treated in the same way with Griess reagent.

Statistical analysis

The Student's *t*-test was used for the free radical scavenging studies, while the Z test was used for survival studies (Abramowitz and Stegun, 1972) using the following formula:

$$z = \frac{\hat{p}_1 - \hat{p}_2}{\sqrt{\hat{p}(1 - \hat{p})(1/n_1 + 1/n_2)}}$$

where $\hat{p}$ = (number of successes)/total sample size

RESULTS

Experiment 1. Radioprotective effect of TE

The radioprotective effect of TE was studied in mice treated with 10 mg/kg for 5 consecutive days before exposure to 7, 8, 9, 10, 11, or 12 Gy of γ-radiation. The animals of the DDW + irradiation group exhibited signs of radiation sickness within 2–4 days after exposure to different doses of γ-radiation in a dose-dependent manner. Exposure to higher radiation doses resulted in earlier appearance of the symptoms of radiation sickness. The main symptoms included irritability, lethargy, reduced food and water intake, diarrhea, watering of eyes, weight loss, ruffling of hair, emaciation, and epillation. Facial edema was also observed in a few animals 1–2 weeks after exposure to 9, 10, 11, or 12 Gy. A few animals also exhibited paralysis and difficulty in locomotion during week 2 after exposure. The severity of the symptoms increased with the radiation dose.

The whole body irradiation of mice to 7 Gy did not induce mortality in either group. However, with increasing doses of radiation, survival declined in a dose-dependent manner: at 11 Gy, no survivors were reported beyond 14 days postirradiation in the DDW + irradiation group (Fig. 1; see part D). With the increase in the exposure dose the onset of mortality was also advanced and the first death was observed on day 3 at 12 Gy irradiation and all animals died by day 9 postirradiation (Fig. 1, see part D). The survival was plotted on to the log, while the exposure dose on the linear scale and the $LD_{50/30}$ was found to be 8.6 Gy for this control group (Fig. 2; see part B).

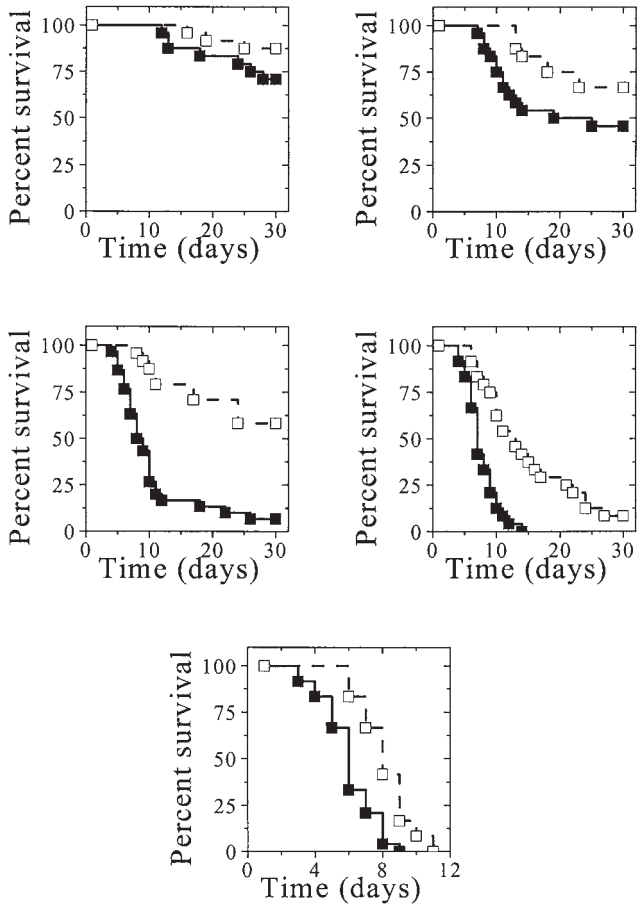

FIG. 1. Kaplan-Meir's estimate of survival of mice treated with 10 mg/kg b. wt of triphala before exposure to various doses of γ-radiation. a: 8, b: 9, c: 10, d: 11 and e: 12 Gy of radiation. Solid squares: DDW + irradiation, open squares: TE + irradiation. Note: All animals of both groups exposed to 7 Gy survived till 30 days and hence the data are not graphically represented.

The pretreatment of mice with 10 mg/kg TE delayed or reduced the severity of radiation sickness and the onset of radiation-induced mortality when compared with the concurrent DDW + irradiation group. The onset of mortality was delayed by approximately 3–4 days in the TE + irradiation group when compared with the DDW + irradiation group. The gastrointestinal (GI) deaths were fewer compared to DDW + irradiation group for all exposure doses (Fig. 2A). Similarly, TE treatment also increased the number of survivors at 30 days post-irradiation ($p < 0.001$ and 0.002 for 9 and 10 Gy, respectively), when compared with the concurrent DDW + irradiation group (Fig. 2B). The $LD_{50/30}$ was found to be 9.9 Gy for the TE + irradiation group, an increase of 1.4 Gy. The dose reduction factor (DRF) was found to be 1.15 (Fig. 2; see part B).

Experiment 2. Free radical scavenging in vitro

The data are shown as percent scavenging of free radicals in Figure 3. The TE inhibited the generation of $^{\bullet}OH$ and $O_2^{\bullet-}$ radicals in a dose-dependent manner and a maximum scavenging was observed at 500 μg/mL (Fig. 3A, B). The total antioxidant activity was measured using ABTS assay and the inhibition of $ABTS^{\bullet+}$ radicals showed a dose-dependent scavenging with a peak observed at 500 μg/mL (Fig. 3C). Similarly, TE also showed a dose-dependent scavenging of NO up to 200 μg/mL; thereafter, NO scavenging declined with increase in the drug concentration (Fig. 3D). TE caused a significant inhibition in the induction of $^{\bullet}OH$, $O_2^{\bullet-}$, $ABTS^{\bullet+}$, and NO free radicals.

DISCUSSION

The Ayurveda extensively uses plant-derived compound formulations for the treatment of various ailments after a careful study into the type of the disease (Sivarajan and Balachandra, 1996). Often the drugs formulated have the desired activity with adequate potency, and are devoid of untoward side effects. The desired activity is rarely present in adequate potency in a single plant, or it may also contain unwanted effects. Therefore, several plants with the com-

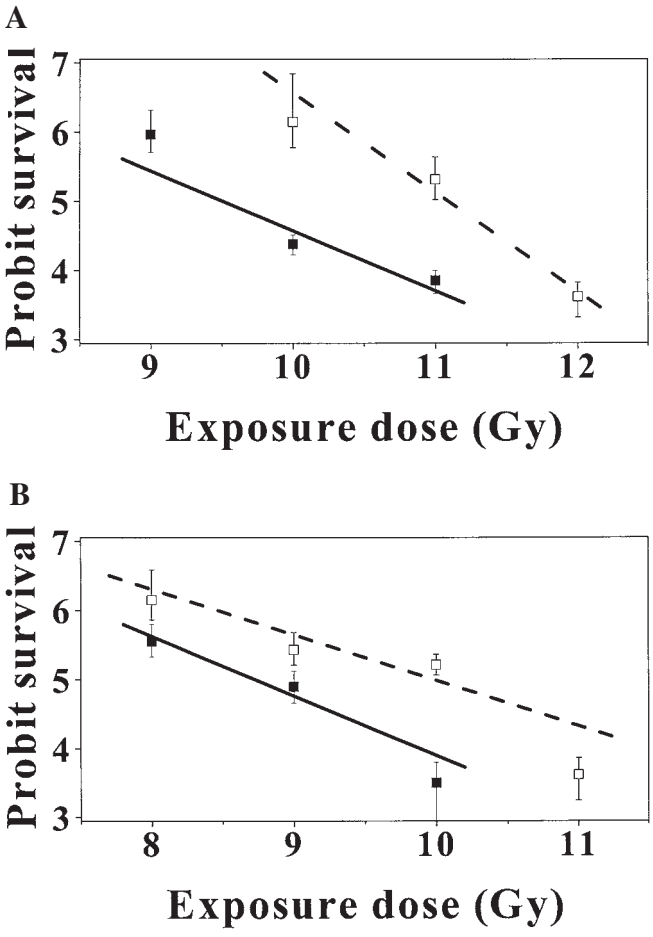

FIG. 2. Alteration in the radiation-induced mortality of mice treated with 10 mg/kg triphala extract: on day 10 and b: on day 30. Solid squares: DDW + irradiation and open squares: TE + radiation.

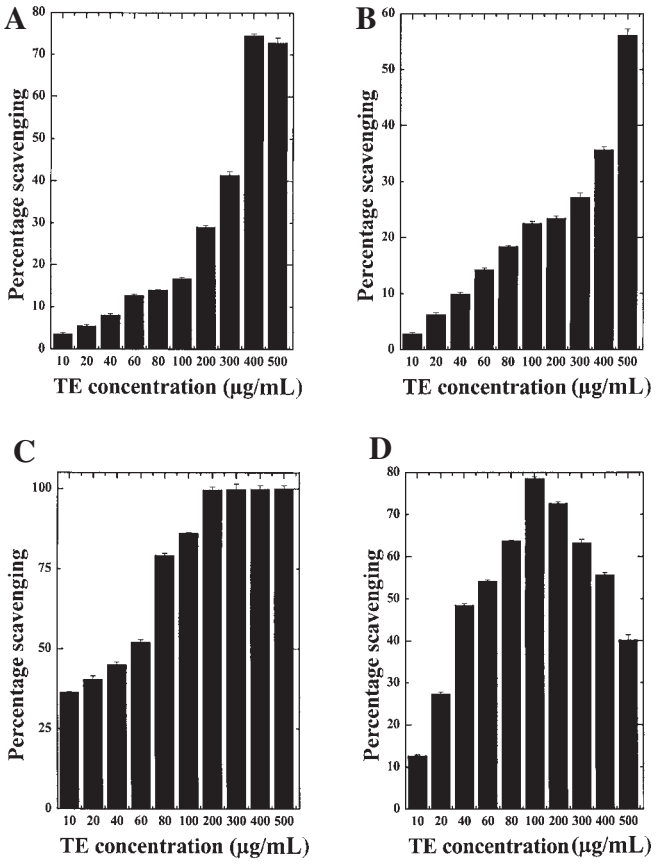

FIG. 3. Effect of various concentrations of triphala extract on the scavenging of various free radicals. (**A**) Hydroxyl (**B**) Superoxide anion, (**C**) $ABTS^{\cdot+}$ cation, and (**D**) Nitric $oxide^{\cdot+}$ radicals.

mon desired activity but differing undesirable activities are selected, so that the final formulation will have a concentrated desired activity and the undesired activities will be absent, or diluted. Further, in such a formulation, certain other compounds may help in enhancing the potency of the active compounds, resulting in an additive or synergistic positive effect, which may be of immense benefit to the patient (Kulkarni, 1997).

Ionizing radiation after whole body exposure produces symptoms of radiation sickness and induces mortality that increases in a dose-dependent manner. A single whole-body exposure of mammals to ionizing radiation results in a complex set of symptoms whose onset, nature, and severity are a function of both total radiation dose and radiation quality. At the cellular level, ionizing radiation can induce damage in the biologically important macromolecules such as the DNA, proteins, lipids, and carbohydrates in various organs. While some damage may be expressed early, some may be expressed over a period of time, depending upon the cell kinetics and the radiation tolerance of the tissues. The proliferating cells are highly sensitive to the effect of radiation; therefore, the effect of whole body irradiation is mainly felt by the highly proliferating germinal epithelium, GI epithelium, and the bone marrow progenitor cells. Of these, the germinal epithelium does not affect the mortality of the exposed individual, while the GI epithelium and the bone marrow progenitor cells are crucial for the sustenance of life; any damage to these cells will impair the normal physiological processes, with a drastically adverse impact on survival. The GI epithelium is less sensitive than the bone marrow progenitor cells, but as the cell transit time is more rapid, it is expressed earlier than the hemopoetic syndrome. In mice, death within 10 days post-irradiation is due to GI damage. The bone marrow stem cells are more sensitive to radiation damage than the intestinal crypt and the hemopoietic syndrome occurs at lower doses and is manifested as hemopoietic stem cell depletion, followed by the depletion of mature hemopoietic and immune cells. However, the peripheral blood cells have a longer transit time than the intestinal cells and, in mice, death from 11 to 30 days post-irradiation is due to the hemopoetic damage inflicted by radiation (Bond et al., 1965; Jagetia and Baliga, 2002, 2003; Jagetia et al., 2002, 2003). A similar effect has been observed in the present study.

Treatment of mice with 10 mg/kg TE before exposure to different doses of gamma radiation protects against the GI as well as hemopoietic deaths, indicating the radioprotective potential of TE. Several herbal preparations such as Liv-52, abana, cystone, and mentat have been reported to protect against radiation-induced sicknesss, mortality, and DNA damage (Saini et al., 1984a; Jagetia and Ganapathi, 1989, 1991; Jagetia and Aruna, 1997; Jagetia and Baliga, 2002, 2003; Jagetia et al., 2003). Certain Ayurvedic formulations belonging to *rasayana* categories have also been reported to reduce lipid peroxidation and leucopenia in mice.

The administration of 10 mg/kg TE resulted in the protection of mice, and this reduction in GI death may also be due to the protection of intestinal epithelium, allowing for proper absorption of nutrients. TE has been used as a laxative to support the body's vitality in man, and it stops diarrhea. Our findings support the contention that TE may protect the GI tract epithelium against the toxic insult of radiation, protecting mice against GI death in this study. *T. chebula*, an important constituent of TE, is reported to improve the GI motility; mitigate cysteamine-induced duodenal ulcers in rats by increasing the beta-glucuronidase activity in the Brunner's glands; and protect the epithelial cells against the cytopathic effects caused by influenza A virus (Badmaev and Nowakowski, 2000; Nadar and Pillai, 1989; Tamhane et al., 1997).

The TE treatment significantly reduced the bone marrow deaths in the TE + irradiation group. The increase in 30 day survival may be due to the protection afforded by TE to the stem cell compartment of the bone marrow, which continued to supply the requisite number of cells in the survivors. Various other plant formulations have been reported to protect against radiation-induced damage in the bone marrow cells (Jagetia and Aruna, 1997; Jagetia and Ganapathi, 1989,

1991; Kumar et al., 1996; Saini et al., 1984a,b). Further, TE and its constituents are reported to possess antimicrobial activity (Ahmad et al., 1998; Dutta et al., 1998; Mehta et al., 1993; Phadke and Kulkarni, 1989; Valsaraj et al., 1997), which could also be responsible for the radioprotective action of TE. One of the constituents of TE, the *P. emblica*, has been found to be an immunomodulator (Rege et al., 1999; Suresh and Vasudevan, 1994) and this would have increased the body defence system by increasing immunity. Maharishi Amrit Kalash (M4), a *rasayana* drug, has been reported to protect humans against the toxicity induced during chemotherapy (Srivastava et al., 2000).

TE is mainly composed of *T. chebula*, *P. emblica*, and *T. bellerica* in equal proportions and each plant has been utilized to treat various ailments and diseases in the Ayurvedic system of medicine. The water and chloroform extracts of *T. chebula* have been shown to protect against sodium azide and 4-nitro-o-phenylenediamine induced mutagenesis (Grover and Bala, 1992). Recently, it has also been reported to possess antioxidant activity and prevent the TPA-induced DNA breaks in human erythrocytes (Naiwu et al., 1992). Similarly, *T. bellerica* has been found to contain anti-HIV-1, antimalarial, and antifungal activity (Valsaraj et al., 1997). The alcoholic extract of this plant has been found to reduce the serum GOT, GPT, and LDH activity, cause a significant reduction in fatty acid levels, and protect against myocardial necrosis (Tariq et al., 1977). *P. embelica* has also been found to be rich in ascorbic acid, and ascorbic acid has been reported to reduce radiation-induced sickness and mortality (Redpath et al., 1982) and to protect mice bone marrow cells against radiation-induced chromosome damage (Sarma and Keshavan, 1993). In addition to ascorbic acid, *P. emblica*, *T. chebula*, and *T. bellerica* also contain ellagic acid, which has been reported to decrease the bone marrow micronuclei formation in mice (Thresiamma et al., 1996). *P. emblica* has also been reported to contain flavonoids (Jose and Kuttan, 1995), a class of compounds reported to possess antioxidant and free radical scavenging activities (Korina and Afanasev, 1997; Tanaka, 1994; Uddin and Ahmad, 1995). Certain flavanoids have been found to protect against radiation-induced DNA damage (Jagetia and Reddy, 2002; Shimoi et al 1994; Shimoi et al., 1996, 1997) and mortality (Uma Devi et al., 1999). The aqueous, acetone, and chloroform extracts of *E. officinale* have been found to be antimutagenic (Grover and Kaur, 1989).

The exact mechanism of action of TE is not known. Since ionizing radiation induces cellular damage by producing a burst of free radicals, the scavenging of free radical may be an important mechanism of radioprotection by TE. This is supported by studies on free radical scavenging activity of TE, which scavenges not only $^{\bullet}OH$, but also other deleterious free radicals such as $O_2^{\bullet -}$, $ABTS^{\bullet +}$, and NO in a dose-dependent manner *in vitro*. There is reason to believe that a similar action of TE is operational *in vivo*. The other plausible mechanism may be the reduction of lipid peroxides, which are reported to induce damage to the cellular genome (Leyko and Bartoss, 1986; Noda et al., 1993; Raleigh et al., 1977) accompanied by elevation of GSH. Another radioprotective herbal formulation, Liv-52, has been reported to reduce radiation-induced lipid peroxidation in mice (Ganapathi and Jagetia, 1995). Recently, ginger has been found to reduce radiation-induced lipid peroxidation and increase the GSH level in irradiated mice (Jagetia et al., 2003). The aqueous extract of one of the constituents of TE, *P. emblica*, has been reported to be a potent inhibitor of lipid peroxidation, scavenging hydroxyl and superoxide radicals *in vitro* (Jose and Kuttan, 1995). Phytochemical studies have shown that *T. bellerica* contains bellericanin, ellagic acid, gallic acid, chebulagic acid, ethyl gallate, and β-sitosterol. *T. chebula* has been found to contain chebulin, terchebin, chebulagic acid, chebulinic acid, corilagin, ellagitannin, ellagic acid, gallic acid, β-D-glucogallin, and terchebin. *P. emblica* has been reported to contain terchebin, corilagin, tannins, ellagic acid, phyllembic acid, gallic acid, and flavonoids in different proportions depending on the season, type of climate, and the plant processing (Chemexcil, 1992; Council for Scientific and Industrial Research 1952, 1976; Satyavati et al., 1987; Jose and Kuttan, 1995; Rastogi and Mehrotra, 1990). Most of these compounds have been reported to possess antioxidant and free radical scavenging activities (Korina and Afanasev, 1997; Tanaka, 1994; Uddin and Ahmad, 1995) and increase the antioxidant enzymes (Kong Ah-Ng et al., 2000). The presence of various antioxidant compounds in TE may also have contributed to its radioprotective action.

CONCLUSION

From our study it is clear that TE provided protection against radiation-induced sickness and mortality in mice and its free radical scavenging and antioxidant activities may be responsible for the observed radioprotection. Further experimental studies are planned to explore how TE may modulate the radiation-induced damage in radiation fractionation, for its utility in cancer treatment and its effect on cancer growth.

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