

Effect of AC II, a herbal formulation on radiation-induced immunosuppression in mice

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A single dose of 6 Gy irradiation significantly reduced the total WBC count while in herbal formulation (AC II) treated groups it was found to be significantly increased. Similarly bone marrow cellularity and α -esterase positive cells, which were lowered by radiation, were partly restored in AC II treated groups. The data indicate that AC II can overcome the immunosuppression produced by irradiation.

Keywords: Herbal drugs, Immunosuppression, Radiation damage, Traditional medicine

Radiation is therapeutically used for the treatment of various types of malignancies. Irradiation produces several biological effects including myelosuppression, immunosuppression, and genetic damage. Ionizing radiation can induce damage in biologically important macromolecules such as DNA, proteins, lipids and carbohydrates in the various organs. The effect of whole body irradiation is mainly felt by the highly proliferating germinal epithelium, gastrointestinal epithelium and the bone marrow progenitor cells. Of these, the germinal epithelium does not have a life supporting function in the exposed individual. But the gastrointestinal epithelium and the bone marrow progenitor cells are crucial for the sustenance of life and any damage to these will drastically impair the normal physiological process¹. Several agents like sulphydryl compounds, antioxidants, metallic salts, metallothionine and immunomodulators have been used to reduce the deleterious effects of irradiation².

Due to the high cost, adverse effects and limitations associated with these agents, herbal medicines have frequently been used as an alternative to medical therapy. In the traditional Indian system of medicine, Ayurveda, herbal formulations are extensively used for the treatment of various diseases.

AC II was formulated at Amala Ayurvedic Research Centre, which contains plant materials with known immunostimulating activity such as *Syzygium aromaticum* (flower buds), *Mesua ferrea* (flower buds), *Elettaria cardamomum* (seeds), *Piper nigrum* (seeds), *Piper longum* (seeds), *Zingiber officinale* (rhizome) and *Withania somnifera* (rhizome)³. AC II was prepared by taking a proportionate quantity of each of these ingredients, which were dried and powdered together. Using this preparation the centre is treating immunosuppression in HIV sero positive individuals (symptomatic and nonsymptomatic patients) for the last 10 years³. There are four aspects of using herbal medicines in the context of HIV infection: to attack the virus, support the immune system, treat or prevent secondary infections, and relieve side effects. We have observed that in symptomatic patients, administration of the drug relieves the symptoms and in the case of unsymptomatic patients the drug delays their onset. AC II was found to have an immunomodulatory effect in normal⁴ and in cyclophosphamide treated animals⁵. The present study has been undertaken on the activity of AC II in improving the immune status in irradiated animals.

Materials and Methods

Chemicals—Harris haematoxylin was purchased from Glaxo India, Ltd., Mumbai. *Para*-rosaniline

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hydrochloride and α -naphthyl acetate were obtained from Loba Chemie, Mumbai. All other chemicals used in these experiments were of analytical reagent grade and purchased locally.

Animals—BALB/c mice (6-8 weeks old) were purchased from Small Animal Breeding Station, Veterinary College, Thrissur. Animals were housed in ventilated cages and were given mouse chow (Sai Durga Foods and Feeds, Bangalore) and water *ad libitum*. All animal experiments were conducted after getting sanction from Institutional Animal Ethics Committee and as per the instructions prescribed by the Committee for the purpose of content and supervisions of experiments on animals (CPCSEA), Ministry of Environment and Forest, Government of India.

Irradiation—Animals were treated with a single dose of radiation of 6 Gy. This dose was selected as it could produce severe immunosuppression in the animals without any mortality⁶. The source of radiation was a ⁶⁰Co Theratron- Phoenix teletherapy unit (Atomic Energy Ltd, Canada). Animals were restrained in specially designed, well-ventilated cages without anesthesia and exposed to whole body radiation at a dose rate of 1.33 Gy/min in a field size of 25×25 cm² and at a distance of 80 cm from the source.

Preparation of drug extract—AC II, herbal powder was prepared at Amala Ayurvedic Research Centre under supervision of qualified persons. Each time, 10 g of AC II was boiled in 500 ml distilled water for 1 hr. Filtrate was evaporated to dryness under vacuum at 50°-55°C using a rotary evaporator under reduced pressure. The yield of the preparation of AC II was 3.5g. Evaporated extracts were reconstituted in water.

Determination of immune competence of AC II in normal animals

a) **Determination of effect of AC II on circulating antibody titre**—BALB/c male were divided into two groups (6 animals/group). Group I animals were kept as untreated control. Group II were fed orally with 5 doses of AC II 1g/kg body weight. Further, all animals were immunized with sheep red blood cells (2.5×10^8 cells). Blood was collected from the tail vein, every third day for 30 days; serum separated and heat inactivated at 56°C. Antibody titre was determined using sheep red blood cells as antigen⁷.

b) **Determination of effect of AC II on antibody forming cells in spleen**—Mice were divided into two groups (12 animals/group). Group I acted as untreated

controls. Group II was fed with 5 doses of AC II. Further, all animals were immunized with sheep red blood cells (2.5×10^8 cells). The animals were sacrificed on different days starting from 3rd day and continued till 14th day after immunization. Spleens were removed, processed to single cell suspension and used to determine the antibody forming cells by Jern's Plaque assay⁸.

Determination of the effect of AC II on hematological parameters—Male mice were divided into following four groups of 6 animals/group:

Group I	Normal
Group II	Irradiation (6 Gy)
Group III	AC II + Irradiation + AC II (250 mg/kg body weight)
Group IV	AC II + Irradiation + AC II (1 g/kg body weight)

All the animals in group II, III and IV were irradiated with a single dose of 6 Gy. AC II extract was given orally to Group III and Group IV. Drug administration was started three days prior to radiation and it was continued for one month. Blood was collected from the caudal vein into heparinized tubes before irradiation and continued every third day for 30 days. Following parameters were checked (a) body weight; (b) total WBC count (haemocytometer method); (c) hemoglobin (cyanomethemoglobin method)⁹; (d) differential count.

Determination of effect of AC II on bone marrow cellularity and α -esterase positive cells—BALB/c mice were divided into following four groups of 15 animals/group:

Group I	Normal
Group II	Irradiation (6 Gy)
Group III	AC II + Irradiation + AC II (250 mg/kg body weight)
Group IV	AC II + Irradiation + AC II (1 g/kg body weight)

All animals were irradiated with a single dose of 6 Gy. AC II (250 mg/kg body weight and 1g/kg body weight) were given orally to Group III and IV respectively for one month in the same protocol as given for previous experiment. Three animals from each group were sacrificed before irradiation and on 3rd, 8th, 16th, 24th and 30th day after irradiation. Bone marrow was collected from both femurs into PBS containing 2% bovine calf serum and made into single cell suspensions. The total number of bone marrow

cells were counted using a haemocytometer and expressed as total live cells $\times 10^6$ /femur¹⁰.

From the above bone marrow preparation smears were made on clean glass slide and stained with *p*-rosaniline and Harris hematoxylin to determine nonspecific α -esterase activity by simultaneous azo dye coupling. Values were expressed as total α -esterase positive cells/4000cells¹¹. Thymus and spleen were excised from the animals, washed thoroughly in ice-cold saline and their weights were recorded.

Statistical analysis—Data were expressed as mean $\pm$ standard deviation (SD). Significance levels for comparison of differences were determined using Student's *t* test.

Results

Effect of AC II on circulating antibody titre and plaque forming cells (PFC)—There was a significant increase in the amount of circulating antibody in animals treated with AC II (Table 1) on various days after antigen administration. The maximum antibody titre (2048) was observed on day 24 after the antigen administration, while in the control the maximum titre (64) was seen on day 12.

AC II also increased antibody forming cells as seen by Jern's plaque assay. The maximum number of plaque forming cells (708 cells/ 10^6 spleen cells) was observed in animals treated with AC II on 8th day, while in control animals it was 181cells/ 10^6 spleen cells on day 6. These results indicated that AC II administration could significantly increase the immunity in these animal models.

Effect of AC II on hematological parameters in irradiated animals—Irradiation did not produce a statistically significant reduction in the body weight of the exposed animals. After 72 hr of irradiation, in

control animals, initial body weight was reduced from 26 ± 1.2 to 25.45 ± 1.54 g. However, on subsequent days weight change in animals did not differ significantly in treated compared to control groups. After one month of drug administration, there was no significant change in body weight in both groups (Table 2).

The total WBC count was significantly reduced from 6785 ± 249 to 3275 ± 890 cells/mm³ at 72 hr after irradiation. Lowered WBC was significantly increased by the administration AC II and on day 15, total WBC of irradiated animals was 4667 ± 852 cells/mm³ while that of AC II animals it was 5100 ± 1038 cells/mm³ respectively (Fig. 1).

There was no significant change in the hemoglobin content of irradiated animals when compared with drug-treated or normal animals (Table 3). Administration of AC II also did not produce any change on differential count especially in lymphocyte-neutrophil ratio (data not presented).

Table 1—Effect of AC II on circulating antibody titre and plaque forming cells

Day	Antibody titre		Day	Plaque forming cells/ 10^6 spleen cells	
	Control	AC II (1g/kg body weight)		Control	AC II (1g/kg body weight)
3	8	64	3	63	111
6	32	128	4	123	217
9	32	128	5	172	286
12	64	256	6	181	408
15	32	256	7	163	600
18	32	512	8	126	708
21	32	1024	9	107	644
24	32	2048	10	56	453
27	16	1024	11	53	378
30	16	512	12	43	240

Table 2—Effect of AC II on body weight in irradiated (6 Gy) animals

[Values are mean $\pm$ SD from 6 animals in each group]

Group	Body weight (g) on different days after radiation								
	-3	3	6	9	12	15	18	21	30
Normal	25.20 ± 2.80	25.05 ± 1.55	25.71 ± 1.98	25.88 ± 2.06	25.80 ± 2.54	25.86 ± 2.55	26.60 ± 2.29	27.35 ± 2.19	27.40 ± 2.03
Irradiation	26.00 ± 1.20	25.45 ± 1.54	25.36 ± 1.53	25.73 ± 1.28	25.88 ± 1.04	26.30 ± 0.61	26.55 ± 1.43	27.48 ± 1.86	28.31 ± 1.80
AC II + IR + AC II 250 mg/kg body weight	25.83 ± 1.77	25.21 ± 2.23	24.75 ± 2.40	25.60 ± 2.52	25.95 ± 2.20	26.70 ± 1.46	26.86 ± 2.10	28.00 ± 1.87	29.06 ± 1.95
AC II + IR + AC II + 250mg/kg body weight	26.13 ± 1.50	25.15 ± 1.40	25.06 ± 1.31	26.03 ± 1.02	26.25 ± 1.61	26.66 ± 1.40	27.00 ± 1.45	27.91 ± 2.00	29.11 ± 1.96

Effect of AC II on bone marrow cellularity and α -esterase activity in irradiated animals—Bone marrow cellularity was found to be reduced from $14 \pm 1.11 \times 10^6$ in normal animals to $6.2 \pm 1.58 \times 10^6$ cells/femur 72 hr after irradiation (Fig. 2). Simultaneous administration of AC II was found to improve bone marrow cellularity significantly. On day 16, bone marrow cellularity of irradiated animals was $6.0 \pm 1.12 \times 10^6$ cells/femur while in AC II treated animals (1 g/kg body weight) it was found to be $8.5 \pm 1.0 \times 10^6$ cells/femur ($P < 0.01$).

α -Esterase positive cells in irradiated animals was found to be significantly reduced from 683 ± 35 to 235 ± 33 cells/4000 cells 72 hr after irradiation (Fig. 3). Simultaneous administration of AC II was found to improve α -esterase positive cells. On day 16, the number of α -esterase positive cells in irradiated animals was 276 ± 29 cells/4000 cells while in AC II treated animals (1 g/kg body weight) it was found to be 499 ± 249 cells/4000 cells ($P < 0.01$).

Organ weights of thymus and spleen of the animals undergoing radiation treatment in presence and

Table 3—Effect of AC II on hemoglobin content in irradiated (6 Gy) animals

[Values are mean $\pm$ SD from 6 animals in each group]

Group	Hemoglobin content (g/dl) on different days after radiation								
	-3	3	6	9	12	15	18	21	30
Normal	14.15 ± 1.43	10.40 ± 1.38	12.52 ± 0.97	10.51 ± 2.72	10.34 ± 0.96	8.47 ± 0.68	8.43 ± 0.94	8.84 ± 0.83	10.67 ± 2.36
Irradiation	9.52 ± 1.2	9.67 ± 1.62	8.62 ± 0.76	10.11 ± 1.77	10.76 ± 1.9	11.18 ± 2.03	9.27 ± 1.54	9.19 ± 1.28	9.47 ± 1.86
AC II + IR + AC II 250 mg/kg body weight	8.77 ± 1.18	9.65 ± 1.12	9.27 ± 1.46	9.05 ± 1.94	9.40 ± 1.18	8.84 ± 1.19	8.85 ± 0.98	8.30 ± 0.76	9.32 ± 1.79
AC II + IR + AC II 1 g/kg body weight	10.51 ± 1.73	9.81 ± 1.94	9.89 ± 1.33	8.69 ± 0.99	9.88 ± 1.13	9.97 ± 1.17	8.94 ± 0.33	9.35 ± 0.71	9.90 ± 1.78

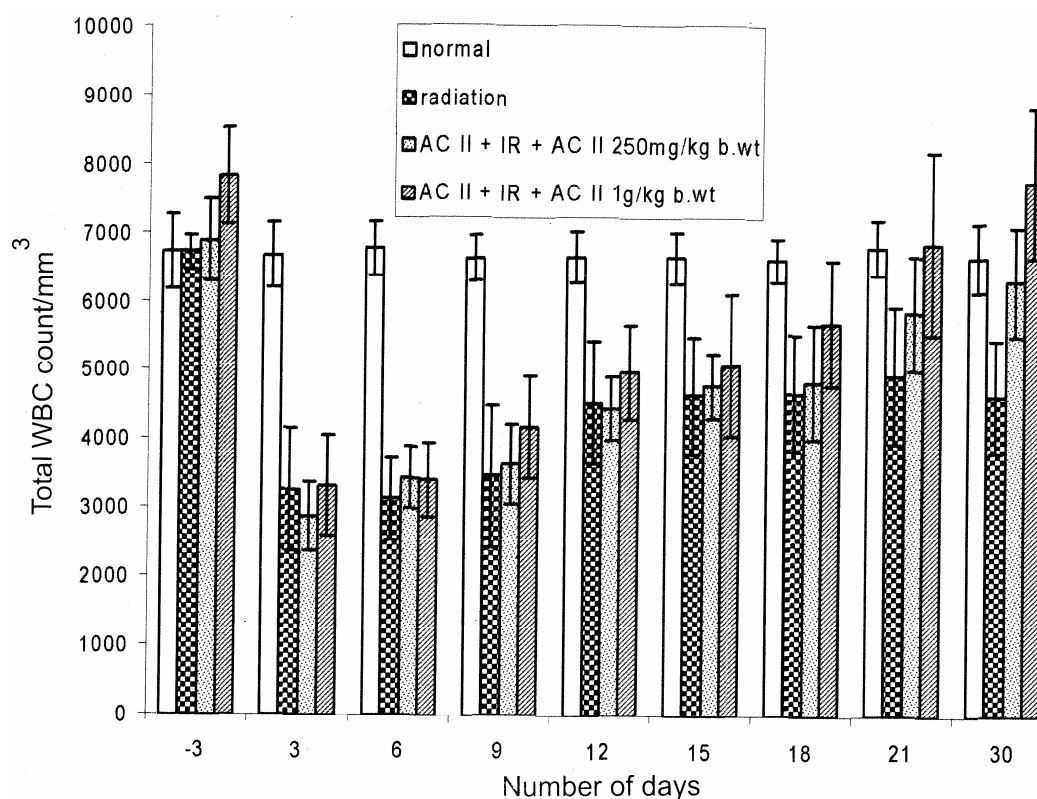


Fig. 1—Effect of AC II on total WBC count in irradiated animals

absence of AC II were also recorded (data not presented). A decrease in thymus weight was observed in radiation treated animals compared to normal animals initially. Treatment with AC II was found to increase the weight of the thymus in irradiated animals. There was no significant change in spleen weights under this condition.

Discussion

Radiotherapy is a regional and cytotoxic therapy. It causes cyto-reduction not only in tumor cells but also in healthy normal tissues that lie within the radiation field. Rapidly dividing cells of gastrointestinal tract, haemopoietic systems are more prone to radiation-induced damages. This may result in impairment of patient's life. Several strategies are designed in order to minimize the lethal consequence of radiation exposure to normal cells. Use of radioprotectors is one among various other strategies². Several compounds such as WR2721, Vitamin C, Vitamin D,

2-mercaptoethanesulfonic acid (MESNA) and several biological modifiers like γ -interferon have been tried as radioprotective agents in experimental animals as well as in humans. The fact remains that to date there is not a single radioprotective agent available which meets all the prerequisites of an ideal radioprotector, i.e., produces no cumulative or irreversible toxicity, offers effective long-term protection, possesses a shelf life of 2-5 years, and can be easily administered¹². Pharmacological activities of radioprotectors may be their antioxidant^{13,14}, anti-inflammatory, immunostimulatory¹⁵, wound healing or antimicrobial activities¹⁶.

Exposure of mammals to ionizing radiation leads to the development of a complex dose dependent cascade of changes including injury to the lymphoid and haemopoietic system, which can result in septicemia and death¹⁷. In the present study, after irradiation, there was a severe immunodepression as seen by a prompt fall in WBC count and bone marrow

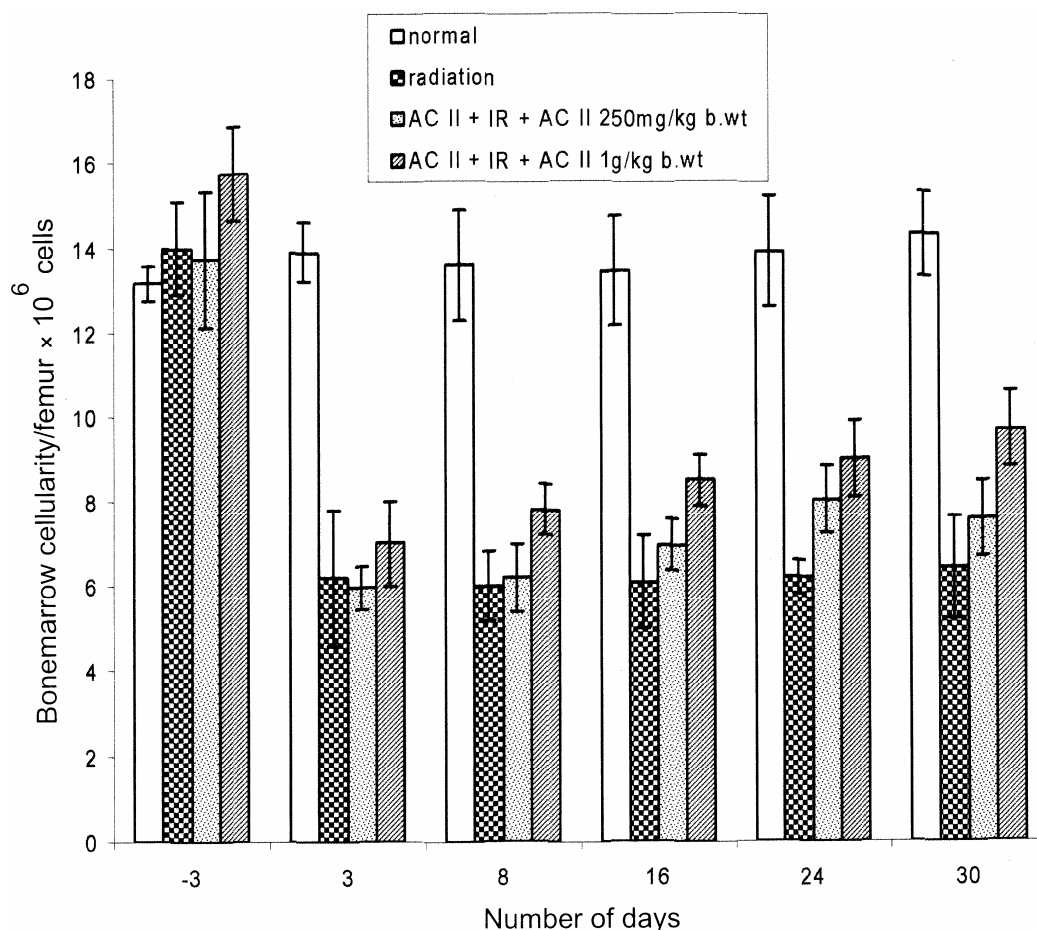


Fig. 2—Effect of AC II on bone marrow cellularity in irradiated animals

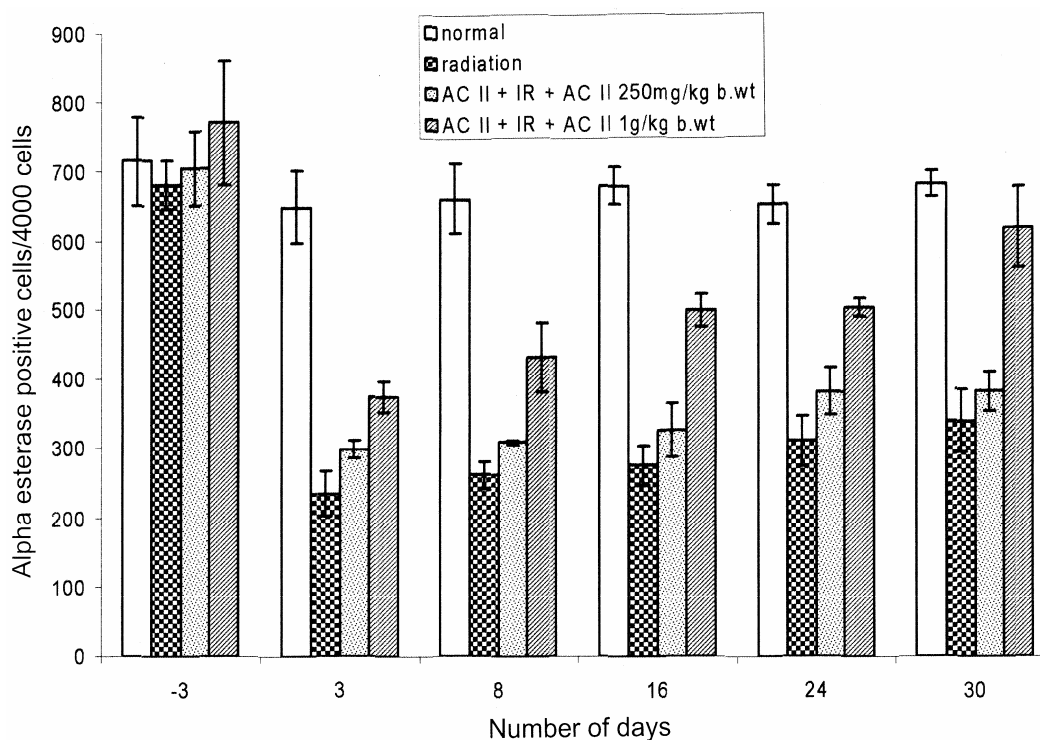


Fig. 3—Effect of AC II on α -esterase activity in irradiated animals

cellularity. Simultaneous administration of AC II enhances the total WBC count and bone marrow cellularity. Agents capable of inducing the haemopoietic system have typically been associated with haemopoietic regeneration.

The repertoire of immune defenses used by the body in defending against diseases involves mainly three effector arms: humoral and cellular immunity and the regulators of the immune system such as cytokines¹⁸. Secreted antibodies and complement components are effectors of humoral immunity. Humoral immune responses were enhanced by the administration of AC II, as evidenced by the increased production of circulating antibody titre and antibody forming cells.

Immunomodulatory agents can enhance or inhibit the immunological responsiveness of an organism by interfering with its regulatory mechanisms¹⁹. Radioprotective activity conferred by immunomodulators may be attributed to their capacity to enhance haemopoietic and immune function²⁰. Bone marrow protection and rapid regeneration after cytotoxic therapy may also be important in protecting the animals from the toxic treatment. The increased level of α -esterase positive cell, which is a marker of maturing monocytes further support this observation.

AC II has several drugs with immunomodulatory activity. One of the major ingredient *Piper longum* has been found to protect the mice from lethal effects of radiation and promotes the recovery of bone marrow cells and leukocytes²¹. Immunomodulatory activity of *Piper longum* and Piperine has also been reported^{22,23}. *Withania somnifera* treatment was found to increase the total white and red blood cells and hemoglobin level in normal and irradiated animals²⁴. This extract was also found to reduce the DNA damages as seen from the decreased micronucleated cells in the bone marrow after radiation treatment in mice²⁵. *Mesua ferrea* offered significant protection to Swiss albino mice when challenged with *Salmonella typhimurium* ATCC 6539²⁶. Antibacterial compound 4-alkyl and 4-phenyl 5,7-dihydroxycoumarins were isolated from *Mesua ferrea*²⁷. Aqueous extract of *Elettaria cardamomum* may have component(s), which protect platelets from aggregation and lipid peroxidation²⁸. Aqueous suspensions of cardamomum have protective effect of colon carcinogenesis²⁹. Amrita Bindu, a salt-spice-herbal mixture (*Piper nigrum*, *Piper longum* and *Zingiber officinale*) exerts antioxidant potential against free radical induced oxidative damage³⁰. An extract (EV. EXT.77) derived from *Zingiber officinale* and *Alpinia galanga* inhibits

the induction of several genes encoding cytokines, chemokines and the inducible enzyme cyclooxygenase-2³¹. Radioprotective activity of *Zingiber officinale* was also reported³². Oral administration of aqueous infusions of *Syzygium aromaticum* can delay the formation of papilloma but also reduce the incidence of papilloma as well as cumulative number of papillomas per papilloma bearing mice³³.

In conclusion, data presented here indicating immunomodulatory activity of AC II indicates that AC II could be highly useful not only to counteract immunosuppression in HIV but also could be of value during radiation – induced damage.

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