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Ayurvedic formulations as therapeutic radioprotectors: preclinical studies on Brahma Rasayana and Chyavanaprash

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Exposure to ionizing radiation reduces the cellular antioxidants and causes damage to genomic DNA. In the mammalian system, this results in various radiation syndromes depending on the radiation dose. Commercially available ayurvedic formulations, Brahma Rasayana (BRM) and Chyavanaprash (CHM) were analysed for their ability to restore the cellular antioxidant status and enhance the repair of radiation-induced DNA damages. The antioxidant status in various tissues of mice was restored when these formulations were orally administered, following whole-body exposure to gamma radiation. Administration of these formulations to 4 Gy whole-body gamma-irradiated mice resulted in faster cellular DNA repair, as revealed from the increased cellular repair index and decrease in the formation of micronucleus. This work suggests the possibility of using BRM or CHM as a therapeutic radioprotector during unplanned, accidental ionizing radiation exposure scenario.

Keywords: Antioxidant, Brahma Rasayana, cellular repair index, Chyavanaprash, DNA damage.

IONIZING radiation and radioisotopes have been used for various diagnostic, therapeutic, industrial and other applications such as military purposes, agricultural crop improvement, generation of nuclear power, etc. Personnel manning the radiation sources are often subjected to low levels of radiation and high dose exposure to radiation may occur due to accidents and during nuclear warfare[1].

There is a need to understand the mechanism of radiation damages and its prevention by effective nontoxic drugs. Agents which reduce the radiation-induced free-radical-mediated damages and enhance the cellular repair and recovery processes are called radioprotectors[2]. Development of novel and effective non-toxic radioprotectors is of considerable interest for application in defence (nuclear wars), nuclear industries, radiation accidents, space flight, etc., besides playing an important role in the protection of normal tissues during radiotherapy of tumours[3]. So far, no appropriate radioprotectors are available for practical applications with acceptable toxicity in the clinical situations. Amifostine, the only FDA-approved and clinically accepted radioprotector[4], is of limited utility due to its severe side effects at clinically

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effective doses[5]. The effectiveness of amifostine as a radioprotector under post-radiation exposure scenario is also not promising[6]. Ionizing radiation is used in a multitude of arenas, and the planned and unplanned exposure to a huge population is a major concern which necessitates the development of non-toxic, easily administrable drugs which can protect against radiation-induced damages.

Ayurveda, the Indian holistic healthcare system of traditional medicine with natural constituents[7], is an untapped source of radioprotectors. Dietary antioxidants have been reported to protect cells from radiation-induced damages[8]. Most of the plants used in ayurvedic drugs have significant antioxidant activity[9,10]. Brahma Rasayana (BRM) is reported to have antioxidant, myeloprotecting, immunostimulatory, anti-inflammatory, radioprotective and antitumour properties[11–18]. Chyavanaprash (CHM), with the major constituent being *Emblica officinalis*, has shown to improve the antioxidant status as well as reduce the generation of free radicals[19]. *Emblica officinalis*[20], *Withania somnifera*[21], *Nelumbium speciosum*[22], *Sesamum indicum*[23], *Cinnamomum tamala*[24], *Vitis vinifera*[25], *Piper longum*[26], *Sida cordifolia*[27], *Terminalia chebula*[28], *Aegle marmelos*, *Ipomoea digitata*, *Phyllanthus niruri*, *Tinospora cordifolia* and *Boerhaavia diffusa*[29] are individually reported to possess antioxidant activity, and these are the constituents used for the preparation of BRM and CHM. The present work is an attempt to explore the possible use of BRM and CHM as radioprotecting formulations under post-radiation exposure scenarios.

BRM and CHM made by a reputed manufacturer were obtained from a retail outlet. All the other chemicals and reagents used in this study were of analytical grade.

Swiss albino mice, 8–10 weeks old and weighing 22–25 g, were obtained from the Small Animal Breeding Section (SABS), Kerala Agricultural University, Thrissur. They were kept under standard conditions of temperature and humidity in the Centre's Animal House Facility. The animals were provided with standard mouse chow (Sai Durga Feeds and Foods, Bangalore, India) and water *ad libitum*. All animal experiments in this study were carried out with the prior approval of the Institutional Animal Ethics Committee (IAEC) and were conducted strictly adhering to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of the Government of India.

Irradiation was carried out using a ^{60}Co-Theratron Phoenix Teletherapy unit (Atomic Energy Ltd, Ottawa, Canada) at a dose rate of 1.88 Gy/min.

Swiss albino mice were divided into six groups of 18 animals each and subjected to the following treatments: (i) 0 Gy + 0.2 ml distilled water; (ii) 4 Gy + 0.2 ml distilled water; (iii) 0 Gy + BRM (2 g/kg); (iv) 4 Gy + BRM (2 g/kg); (v) 0 Gy + CHM (2 g/kg) and (vi) 4 Gy + CHM (2 g/kg).

The animals were exposed to 4 Gy whole-body gamma radiation at a dose rate of 1.88 Gy/min and were administered with either BRM and CHM. Administration of these formulations was continued till the 11th day. All animals were sacrificed as described below.

- Two animals were sacrificed from each group at a time interval of 30 min. Bone marrow cells and peripheral blood leukocytes of these animals were subjected to alkaline comet assay.
- At 24 and 48 h of radiation exposure, blood was obtained by tail veining to perform micronucleus assay.
- Four animals from each group were sacrificed on the third day of irradiation. Bone marrow cellularity was monitored. Various tissues were excised out and assayed for different antioxidant parameters such as glutathione (GSH), glutathione peroxidase (GPx) and superoxide dismutase (SOD) and levels of lipid peroxidation.
- Bone marrow cellularity and white blood cell (WBC) count were examined on all alternating days, till the 11th day.

Blood was collected by heart puncture into heparinized tubes for determination of different hematological parameters using an automated hematological analyser unit. The bone marrow was collected from femur into phosphate buffered saline (PBS) containing 2% foetal bovine serum (FBS) and bone marrow cellularity was determined using a hemocytometer.

A small portion of the intestine was fixed in 10% formalin solution, passed through ascending grade of alcohol, cleared in xylene, impregnated and embedded in paraffin. The 3–4 μm sections were made using a microtome and stained with hematoxylin and eosin and observed using a light microscope.

Various tissues such as liver, kidney, intestine and brain were excised and washed with ice-cold PBS. Tissue homogenates 10% (w/v) were prepared in PBS. Level of GSH was assayed by the method of Moron *et al.*[30], based on the reaction with DTNB. GPx activity was measured based on the method of Hafeman *et al.*[31], based on the degradation of H_2O_2. Activity of SOD was measured by NBT reduction method of Mc Cord and Fridovich[32]. Protein level in the tissue was measured following the method of Lowry[33].

The level of peroxidation of membrane lipids was measured by following the method of Buege and Aust[34]. Peroxidation of membrane lipids results in the formation of malondialdehyde (MDA). The amount of MDA present in the homogenate was measured by its reaction with thiobarbituric acid and by measuring the absorption at 532 nm.

Alkaline single-cell gel electrophoresis was performed using the method of Singh[35], with minor modifications[36]. Microscopic slides were coated with normal-melting-

Table 1. Effect of administration of Brahma Rasayana (BRM) and Chyavanaprash (CHM; 2 g/kg) on bone marrow cellularity of mice exposed to 4 Gy gamma radiation

	Day 1	Day 3	Day5	Day 7	Day 11
0 Gy	15 ± 6.9	15 ± 6.9	15 ± 6.89	15 ± 6.9	15 ± 6.9
4 Gy	6.67 ± 3.3	6.67 ± 2.06	6.25 ± 2.9	8.75 ± 3.06	9.55 ± 2.16
0 Gy + BRM	15.91 ± 5.3^{ns}	15.90 ± 5.44^{ns}	16.25 ± 5.6^{ns}	17.25 ± 7.27^{ns}	14.55 ± 4.8^{ns}
4 Gy + BRM	12.73 ± 2.9*	12.73 ± 1.5**	12.75 ± 1.7*	14.33 ± 4.16^{ns}	16.81 ± 3.21*
0 Gy + CHM	15.91 ± 0.64^{ns}	15.91 ± 4.80^{ns}	16.8 ± 3.9 ^{ns}	17 ± 5.65^{ns}	16.25 ± 5.03^{ns}
4 Gy + CHM	13.19 ± 0.64*	13.18 ± 1.41**	12.0 ± 1.0^{ns}	13.5 ± 3.4^{ns}	15 ± 3.67^{ns}

**P < 0.01; *P < 0.05 and ns, 'Not significant' when compared with respective control.

Table 2. Changes in glutathione levels (nmol/mg protein) in different tissues of 4 Gy whole-body-irradiated mice administered with BRM and CHM (2 g/kg)

	Brain	Liver	Intestine	Kidney
0 Gy	127.66 ± 6.87	25.89 ± 3.11	210.65 ± 37.89	42.11 ± 5.32
0 Gy + BRM (2 g/kg)	122.19 ± 24.18^{ns}	24.21 ± 3.48^{ns}	208.2 ± 20.08^{ns}	37.31 ± 6.93^{ns}
0 Gy + CHM (2 g/kg)	130.91 ± 10.21^{ns}	24.11 ± 0.79^{ns}	211.21 ± 26.21^{ns}	34.98 ± 0.81^{ns}
4 Gy	64.52 ± 1.73	15.13 ± 0.81	136.49 ± 39.98	24.35 ± 0.71
4 Gy + BRM (2 g/kg)	81.31 ± 5.22***	19.91 ± 2.09***	183.12 ± 31.9***	30.2 ± 4.09***
4 Gy + CHM (2 g/kg)	79.11 ± 7.27***	21.52 ± 0.94***	185.32 ± 16.53**	27.78 ± 0.68**

***P < 0.001; **P < 0.01; *P < 0.05 and ns, 'Not significant' when compared with respective control.

point agarose (1% in PBS) and kept at 4°C till the agarose was solidified. Next 200 µl of 0.8% low-melting-point agarose containing 50 µl of treated cells were added to the slides. After solidification of the low-melting agarose, the slides were immersed in pre-chilled Lysing solution containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl (pH-10), 1% DMSO, 1% TritonX and kept for 1 h at 4°C for lysis of the cells. After lysis, the slides were drained properly and placed in a horizontal electrophoretic apparatus filled with freshly prepared electrophoresis buffer containing 300 mM NaOH, 1 mM EDTA, 0.2% DMSO (pH ≥ 13). The slides were equilibrated in buffer for 20 min and electrophoresis was carried out for 30 min at 20 V. After electrophoresis the slides were washed gently with 0.4 mM Tris-HCl buffer (pH 7.4) to remove alkali. The slides were again washed with distilled water, kept at 37°C for 2 h to dry the gel and silver-staining was carried out. The comets were visualized under a binocular microscope and the images captured were analysed using the software 'CASP' to find out the extent of DNA damage measured in terms of different comet parameters such as percentage of DNA in tail, tail length, tail moment (TM) and olive tail moment (OTM)[37]. TM is the product of tail length and percentage of DNA in the tail and OTM is the product of the distance between the centre of gravity of the head and the centre of gravity of the tail and percentage of DNA in the tail. Results are presented as mean ± SD.

The micronucleus assay with mouse peripheral blood reticulocytes as reported by Hayashi *et al.*[38] using acridine orange (AO) was carried out to evaluate the chromosomal damage. Peripheral blood was collected from mice by tail vein puncture without any anticoagulant at 24th and 48th h of irradiation onto AO-coated slides, covered immediately with coverglass and these slides were allowed to stand for a few hours or overnight in a refrigerator to allow the cells to settle and to maximize staining. The slides were observed under a blue excitation (488 nm) and a yellow-to-orange barrier filter (515 nm). They were observed for 2000 reticulocytes of peripheral blood (identified by their reticulum structure with red fluorescence) and percentage of micronucleated (round in shape with a strong yellow–green fluorescence) reticulocytes was scored.

The results are presented as mean ± SD of the studied groups. Statistical analyses of the results were performed using ANOVA with Tukey–Kramer multiple comparisons test.

Bone marrow cellularity and total WBC count were found to be significantly reduced at 24 h of 4 Gy gamma radiation exposure. Administration of BRM or CHM significantly prevented radiation-induced decrease in total WBC count. These animals also showed fast recovery of the haematopoietic system as evident from the increase in bone marrow cellularity in subsequent days after radiation exposure (Table 1).

Whole-body gamma irradiation resulted in severe damage to the structure of villi and the interior of the small intestine. In animals administered with BRM or CHM, radiation-induced disruption of villi and damage to intestinal crypt were prevented indicating the radioprotective efficacy of these drugs towards radiation-induced gastrointestinal damage.

Exposure of animals to whole-body gamma radiation (4 Gy) resulted in the depletion of various antioxidant

Table 3. Changes in glutathione peroxidase levels (units/mg protein) in different tissues of 4 Gy whole-body-irradiated mice administered with BRM and CHM (2 g/kg)

	Brain	Liver	Intestine	Kidney
0 Gy	53.12 ± 17.67	23.48 ± 2.97	221.44 ± 41.18	52.8 ± 15.44
0 Gy + BRM (2 g/kg)	53.17 ± 13.62^{ns}	21.56 ± 1.57^{ns}	211.55 ± 36.4^{ns}	53.86 ± 11.46^{ns}
0 Gy + CHM (2 g/kg)	55.13 ± 16.03^{ns}	22.05 ± 2.58^{ns}	209.07 ± 31.17^{ns}	56.14 ± 10.8^{ns}
4 Gy	29.22 ± 4.68	14.47 ± 0.47	93.03 ± 8.97	31.6 ± 1.12
4 Gy + BRM (2 g/kg)	38.56 ± 12.74*	18.06 ± 1.72**	191.83 ± 27.62*	40.84 ± 5.76*
4 Gy + CHM (2 g/kg)	42.37 ± 9.64**	19.54 ± 0.65***	184.02 ± 29.6*	47.08 ± 6.29***

***P < 0.001; **P < 0.01; *P < 0.05 and ns, 'Not significant' when compared with respective control.

Table 4. Changes in superoxide dismutase levels (units/mg protein) in different tissues of 4 Gy whole-body-irradiated mice administered with BRM and CHM (2 g/kg)

	Brain	Liver	Intestine	Kidney
0 Gy	1.26 ± 0.10	13.01 ± 0.26	6.54 ± 0.41	1.18 ± 0.37
0 Gy + BRM (2 g/kg)	1.14 ± 0.09^{ns}	13.06 ± 0.14^{ns}	6.31 ± 1.85^{ns}	1.12 ± 0.12^{ns}
0 Gy + CHM (2 g/kg)	1.17 ± 0.17^{ns}	12.14 ± 0.87^{ns}	6.49 ± 1.68^{ns}	1.12 ± 0.27^{ns}
4 Gy	0.36 ± 0.03	7.9 ± 0.34	2.37 ± 0.58	0.29 ± 0.05
4 Gy + BRM (2 g/kg)	0.81 ± 0.10***	10.2 ± 2.4***	4.18 ± 1.3**	0.82 ± 0.24***
4 Gy + CHM (2 g/kg)	0.77 ± 0.02***	9.49 ± 1.39***	4.19 ± 1.71**	0.88 ± 0.3***

***P < 0.001; **P < 0.01; *P < 0.05 and ns, 'Not significant' when compared with respective control.

Table 5. Changes in lipid peroxidation levels (nmol/mg protein) in different tissues of 4 Gy whole-body-irradiated mice administered with BRM and CHM (2 g/kg)

	Brain	Liver	Intestine	Kidney
0 Gy	10.04 ± 1.44	1.33 ± 0.36	3.05 ± 1.74	5.92 ± 2.8
0 Gy + BRM (2 g/kg)	13.99 ± 2.53^{ns}	1.52 ± 0.47^{ns}	3.04 ± 0.61^{ns}	4.58 ± 1.14^{ns}
0 Gy + CHM (2 g/kg)	14.59 ± 2.26*	1.25 ± 0.28^{ns}	2.85 ± 2.08^{ns}	9.5 ± 1.39^{ns}
4 Gy	33.27 ± 4.34	1.93 ± 0.14	7.75 ± 2.58	37.42 ± 9.55
4 Gy + BRM (2 g/kg)	20.74 ± 5.68***	1.75 ± 0.66***	4.61 ± 1.71***	23.3 ± 5.22***
4 Gy + CHM (2 g/kg)	16.64 ± 2.26***	1.62 ± 0.39***	5.26 ± 0.74***	20.96 ± 4.61***

***P < 0.001; **P < 0.01; *P < 0.05 and ns, 'Not significant' when compared with respective control.

defence systems. The levels of GSH, GPx and SOD in various tissues were significantly reduced (Tables 2–4). In animals administered with BRM or CHM, radiation-induced depletion in antioxidant level was not significantly discernible. The peroxidation of membrane lipids was found to be considerably increased in different tissues of whole-body 4 Gy irradiated animals, while the administration of BRM or CHM prevented the radiation-induced lipid peroxidation (Table 5).

The effect of BRM or CHM on repair of gamma radiation-induced cellular DNA damage was determined by examining various comet parameters such as percentage of DNA in tail, tail length, TM and OTM of blood leukocytes and bone marrow cells of mice exposed to 4 Gy gamma radiation.

To quantify the efficiency of the cells to repair and rejoin strand breaks in DNA, a relation based on the comet parameters of cellular DNA named as cellular DNA repair index (CRI) is used[39]. CRI for a particular comet parameter at any time-point is defined as the percentage decrease from the initial value due to repair.

$$\text{CRI} = [1 - (\text{comet parameter at time } t/\text{comet parameter at initial time } t_0)] \times 100.$$

As the rate of decrease in the comet parameters is attributed to cellular DNA repair, the efficiency of the cells to repair DNA strand breaks following different treatments can be quantified by determining CRI. Data on CRI determined from the comet parameters of bone marrow cells and peripheral blood leukocytes of mice exposed to 4 Gy gamma irradiation are presented in Figures 1 and 2. From the figures it is clear that the administration of BRM or CHM enhanced the efficiency of cells to repair the radiation-induced genomic DNA damage.

Administration of BRM or CHM immediately after 2 Gy whole-body gamma irradiation resulted in a decrease in the percentage of micronucleated reticulocytes (Table 6).

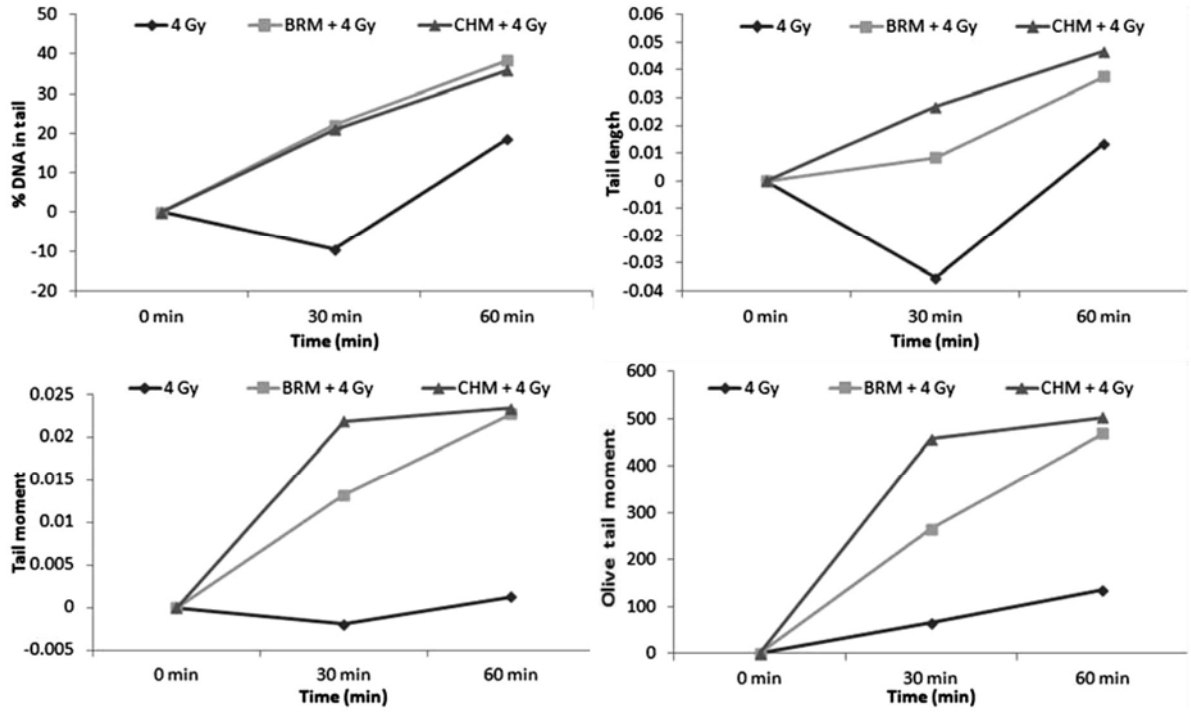

Figure 1. Effect of Brahma Rasayana (BRM) and Chyavanaprash (CHM) (2 g/kg) on repair of cellular DNA of mouse bone marrow cells from damages induced by exposure to 4 Gy gamma radiation expressed as cellular DNA repair index (CRI).

Exposure of cells to ionizing radiation results in damages to cellular DNA[40], peroxidation of membrane lipids[41] and depletion of cellular antioxidant status[42]. In a mammalian organism, total-body exposure to ionizing radiation can result in multiple-organ dysfunctions as a consequence of radiation toxicity to the hematopoietic, gastrointestinal or cerebrovascular systems, depending on the dose of radiation absorbed[43,44]. The hematopoietic and gastrointestinal systems are the most radiosensitive systems and their damage results in the development of hematopoietic syndrome and gastrointestinal syndrome respectively, and mortality of the organism.

Whole-body exposure of animals to sublethal doses (4 Gy) of gamma radiation caused damage to the hematopoietic and gastrointestinal systems and post-irradiation administration of BRM or CHM prevented the manifestation of radiation-induced damage to the hematopoietic and gastrointestinal systems. Administration of these formulations also facilitated the recovery of the cellular antioxidant system and prevented the peroxidation of membrane lipids.

Alkaline single-cell gel electrophoresis or comet assay and micronucleus assay were performed to study the mechanistic aspects of therapeutic post-irradiation protection offered by BRM or CHM. Comet assay is a sensitive, rapid and simple technique for the evaluation of DNA damage and repair[45,46] in terms of double or single-strand breaks. The comet parameters of cellular DNA were found to be significantly increased following whole-body exposure of animals to gamma radiation. These parameters were found to decrease with time because of rejoining of the broken DNA strands due to cellular DNA repair. Administration of animals with either BRM or CHM following whole-body gamma irradiation showed a decrease in these parameters at a faster rate, indicating enhancement in DNA repair. Correspondingly, the CRI in blood leucocytes and bone marrow cells of these animals was found to increase rapidly compared to the control animals, indicating the enhanced DNA repair. The repair of strand breaks measured by comet assay may also involve misjoined double strand breaks (DSBs). These unrepaired DNA strand breaks will result in the formation of chromosomal fragments which fail to join with the genome during mitosis and these fragments will be expressed as micronuclei after cell division[47]. Ionizing radiation-induced micronucleus may arise from DSBs in DNA. Post-irradiation administration of BRM or CHM resulted in a decrease in the frequency of micronucleated reticulocytes, suggestive of an efficient DNA repair mechanism. Thus the results of comet assay and micronucleus assay indicated that these formulations enhanced the rate of repair of damage inflicted by gamma radiation upon the genomic DNA, thereby offering protection to the animals from radiation-induced systemic insults, when administered orally, following radiation exposure.

Radiation therapy is the most common treatment modality for a variety of human cancers for either curative or palliative purpose. One of the limitations for the

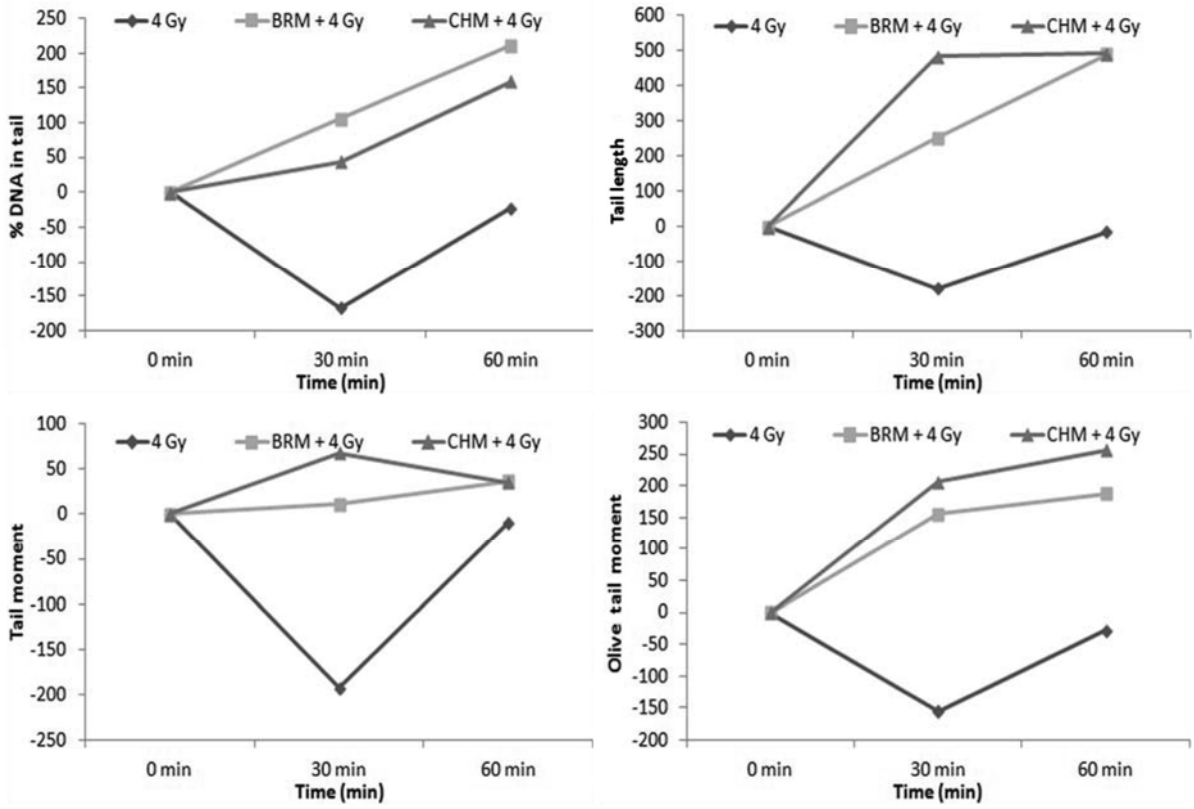

Figure 2. Effect of BRM and CHM (2 g/kg) on repair of cellular DNA of mouse leukocytes from damages induced by exposure to 4 Gy gamma radiation expressed as cellular DNA repair index (CRI).

Table 6. Effect of oral administration of BRM and CHM (2 g/kg) on induction of micronuclei in mouse reticulocytes after exposure to 4 Gy whole-body gamma radiation

Treatment	Micronucleated reticulocyte (%)	
	24 h	48 h
0 Gy	0.8 ± 0.03	0.8 ± 0.03
0 Gy + BRM	0.68 ± 0.06^{ns}	0.8 ± 0.03^{ns}
0 Gy + CHM	0.8 ± 0.03***	0.71 ± 0.08***
4 Gy	3.5 ± 0.56	6.55 ± 0.35
4 Gy + BRM	2.1 ± 0.14^{ns}	4.35 ± 0.35^{ns}
4 Gy + CHM	2.05 ± 0.21***	4.8 ± 0.14***

***P < 0.001 and ns, 'Not significant' when compared with respective control.

success of cancer radiotherapy is the severe side effects and damage to normal tissues in proximity to a tumour[1]. In radiotherapy of cancer, normal tissues need to be protected while cancer cells are exposed to high doses of radiation. An agent producing differential radiation response in tumour and normal tissues would be of great importance in the treatment of cancer by radiation therapy[1]. The only FDA-approved drug exhibiting differential radiation response in tumour and normal tissues and used as an adjuvant in cancer radiotherapy is amyfostin; its use is also limited by severe side effects[1]. Though in preclinical level a number of phytoceuticals, extracts of many plants and medicinal mushroom, *Ganoderma lucidum* have been reported to confer differential radiation response in tumour and normal tissues, an adjuvant effective in radiotherapy is yet a dream[2–5,48]. The activity of enhancing rate of repair could have a role in radiation therapy. CHM and BRM are useful in radiotherapy only if they manifest enhanced rate of DNA repair in normal tissues and not in the tumour tissues. It needs to be ascertained whether there is differential rate of DNA repair enhancement in tumour and normal cells by CHM and BRM. Further studies are needed to establish whether CHM and BRM cause differential effects with respect to DNA repair and radioprotection in normal tissues and tumour. The present work concerns only post-radiation exposure scenarios and hence was particularly focused on normal tissues. It is worth investigating whether CHM and BRM can provide differential radiation response in tumour and normal tissues. Further studies are needed in this direction.

The development of an effective radioprotector useful under post-radiation exposure scenario is important in view of its potential application during unplanned or accidental radiation exposure. During nuclear emergencies, arising from nuclear accidents like those in Three Mile Island, 1979 or in Chernobyl, 1986, or the recent

nuclear crisis due to the tsunami in Japan, there is likelihood of a large population getting exposed to higher doses of radiation. The management and containment of nuclear waste as well as dissemination of proper care to the exposed population will be a Herculean challenge. In such an eventuality, over-the-counter availability of a non-toxic, orally administrable, therapeutic radioprotector will be of immense advantage. The present study revealed the potential of our traditional ayurvedic formulations in combating such challenging situations. BRM or CHM could be directly recommended as a radioprotector without any further clinical trials, since their safety in human beings is time-tested.

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