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Research Paper

Protection of DNA and membranes from gamma-radiation induced damages by *Centella asiatica*

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Abstract

Objectives The objective of the present study was to examine the ability of *Centella asiatica* extract to offer protection to DNA and membranes against the deleterious effects of ionizing radiation exposure.
Methods Protection of DNA under in-vitro conditions of irradiation was estimated using plasmid relaxation assay. For in-vivo studies the extract was administered orally to mice exposed to whole-body γ-radiation. The ability of the extract to offer protection against whole-body γ-radiation exposure was analysed by performing an alkaline comet assay on mouse bone marrow cells. The extent of lipid peroxidation was estimated using the TBARS (thio-barbituric acid reacting substances) method, in order to monitor membrane damage. Radiation-induced mortality of the animals following a lethal dose of γ-radiation was also examined.
Key findings *Centella asiatica* extract significantly reduced radiation-induced damage to DNA. The extent of radiation-induced mortality and lipid peroxidation was also found to be considerably reduced in animals administered with the extract.
Conclusions *Centella asiatica* rendered radioprotection to DNA and membranes against radiation exposure, both *in vitro* and *in vivo*. We have earlier reported that administration of the extract can prevent a radiation-induced decline in antioxidant enzyme levels. This suggests that radioprotection by *Centella asiatica* extract could be mediated by mechanisms that act in a synergistic manner, especially involving antioxidant activity.
Keywords *Centella asiatica*; comet assay; DNA damage; γ-radiation; oxidative stress

Introduction

With the widespread use of nuclear materials for therapeutic purposes, the production of energy and in nuclear industries, there is a need to have effective means to protect not only special high-risk groups but also the general population from the health hazards caused by unintended ionizing radiation exposure. Ionizing radiation induces a wide range of molecular lesions in mammalian cells that can lead to diverse cellular responses such as cell inactivation, chromosomal rearrangements and mutations, eventually resulting in cancer and hereditary diseases. Ionizing radiation generates reactive oxygen species in a biological system by radiolysis of water. Examples include the superoxide anion (O_2^-), the hydroxyl radical ($OH^{\cdot}$), singlet oxygen ($O^{\cdot}$), nitric oxide (NO), hydrogen peroxide (H_2O_2) and peroxyl radicals. These reactive oxygen species can damage several cellular components and biomolecules such as DNA, proteins, lipids, amino acids and carbohydrates.[1]

An unfulfilled dream has been to have a globally effective pharmacological agent that could be easily taken orally, without any undue side effects, prior to a suspected or impending nuclear/radiological event. Such an ideal radioprotective agent has yet to be identified, let alone fully developed and approved for human use. Although the US Food and Drug Administration (FDA)-approved drug amifostine, a thiol phosphate compound, is currently used clinically, its toxicity, limited extent of protection and unfavourable routes of administration all serve to limit its utility in nonclinical settings.[2]

The ability of antioxidants to reduce the cellular damage induced by ionizing radiation has been studied in animal models for more than 50 years. On exposure to ionizing radiation a variety of symptoms are manifested, depending on the dose of exposure. There may be immediate effects or delayed ones. The general complications arising due to ionizing radiations are damage to the haematopoietic system, the gastrointestinal (GI)

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system and the central nervous system (CNS). Depending on the dose of radiation exposure this damage manifests as haematopoietic syndrome, GI syndrome and CNS syndrome.

The uses of plants in traditional medicine are widespread and still serve as leads for the development of novel pharmacological agents. As several of the symptoms of radiation syndromes have some features in common with diseases cured by medicinal plants, it was considered worthwhile to examine the ability of plant products and their compounds to protect biological systems from ionizing radiation.

The present study focuses on the radioprotective property of *Centella asiatica* (Linn), which belongs to the family Umbelliferae, an ethnomedicinal plant used in different continents by ancient diverse cultures and tribal groups. In India, it is usually referred to by the name 'Mandukaparni' in the Ayurvedic system of medicine.[3] In pharmacological studies the plant has shown central nervous system (CNS)-depressant and antitumour activities[4,5] and an inhibitory effect on the biosynthetic activity of fibroblast cells.[6] It has also been shown to be very effective in promoting learning and memory.[7] The extract of *Centella asiatica* has been reported to comprise a mixture of four related triterpenoids: asiatic acid, madecassic acid, asiaticoside and madecassoside.[8] The aim of this study was to characterize the radioprotective activity of *Centella asiatica* by using γ-rays as an oxidative DNA-damaging agent, and to investigate any reduction in the frequency of DNA damage caused by the presence of the extract.

Materials and Methods

Chemicals

Nitroblue tetrazolium, riboflavin, reduced glutathione (GSH) and 5-5′-dithiobis-2-nitrobenzoic acid were obtained from Sigma Chemical Company Inc., St Louis, MO, USA. EDTA was from Sisco Research Laboratories Pvt. Ltd, Mumbai, India. Hydrogen peroxide was from Merck India Ltd, Mumbai, India. Thiobarbituric acid and fetal bovine serum (FBS) were purchased from Hi-media Laboratories, Mumbai, India. Plasmid pBR322 was obtained from Bangalore Genei. All other chemicals and reagents used in this study were of analytical grade procured from reputed Indian manufacturers.

Animals

Male Swiss albino mice, 6–8 weeks old (body weight 20–25 g) were purchased from the Small Animal Breeding Station of Kerala Agricultural University, Mannuthy, Thrissur, and Kerala, India, kept under standard conditions of temperature and humidity, and provided with standard mouse chow and water *ad libitum*. All animal experiments were approved by the Institutional Animal Ethics Committee and were conducted strictly adhering to the ethical guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals, constituted by the Animal Welfare Division, Government of India.

Exposure to γ-radiation

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.

Preparation of *Centella asiatica* extract

Authenticated total plant material of *Centella asiatica* was obtained from the Amala Ayurvedic Hospital and Research Centre, shade dried, powdered and extracted with 70% ethanol at room temperature. The extract was filtered through Whatmann No. I filter paper and the supernatant was evaporated using a rotary evaporator at 45°C and the final liquid suspension was lyophilized to get a powder with a yield of 17% under experimental conditions. Maximum tolerance dose was investigated on the basis of acute toxicity and even a dose of 500 mg/kg body weight did not show any toxic symptoms.[9] The lyophilized powder is hereafter referred to as CAE (*Centella asiatica* extract). The powder was dissolved in distilled water to obtain the desired concentrations. The high performance thin layer chromatography (HPTLC) fingerprinting of the extract was carried out with the solvent system butanol/acetic acid/water (5 : 1 : 4). The HPTLC profile showed a number of components and one of the components (Peak 5 in Figure 1) was identified as asiaticoside in comparison with an authentic sample.

Effect of *Centella asiatica* on γ-radiation induced damage to plasmid DNA *in vitro*

The plasmid pBR322 (150 ng) in phosphate buffer (0.1 M, pH 7.4) was exposed to various doses of γ-irradiation (0–25 Gy) in the presence and absence of CAE (0–10 mg/ml) on ice. After irradiation, DNA was electrophoresed on 0.8% agarose at 55 V for 2 h and the DNA damage was analysed by Digital Gel Documentation and Analysis Software, Biotech R&D Laboratories, Yercaud.[10] The analysis was also performed.[11]

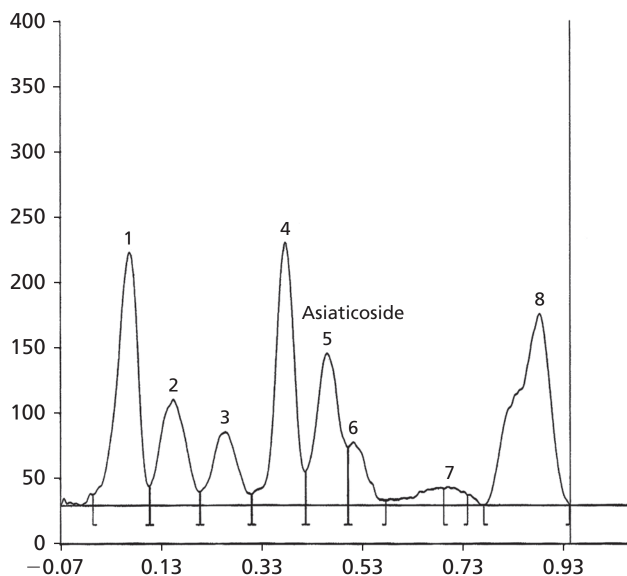

Figure 1 HPTLC profile of *Centella asiatica* extract

Effect of *Centella asiatica* extract on damage to cellular DNA and membrane lipids following γ-radiation under in-vivo conditions

Male Swiss albino mice weighing 20–25 g were divided into four groups, with six animals in each group. The following treatments were given: Group I, double distilled water (DDW) and sham irradiation; Group II, DDW and 4 Gy irradiation; Group III, 200 mg/kg body weight CAE and sham irradiation; Group IV, 200 mg/kg body weight CAE and 4 Gy irradiation.

Animals were sacrificed by cervical dislocation within 1 h of irradiation. Bone marrow cells were collected by flushing the femur bones of each animal with phosphate-buffered saline (PBS) containing 10% FBS. The tissues, such as liver, brain, kidney, spleen and GI mucosa, were excised and the samples were stored in ice until required for further processing. Alkaline comet assay was performed using bone marrow cells.

Alkaline single-cell gel electrophoresis using mouse bone marrow cells (comet assay)

Alkaline comet assay was performed using the method given by Nair and Salvi with minor modifications.[12] Plain microscopic (Blue Star, Mumbai, India) glass slides were coated with normal melting point agarose (1% in PBS), immediately coverslipped and kept at 4°C for 10 min to allow the agarose to solidify. After removal of the cover slip, 200 μl of 0.8% low melting point agarose containing 50 μl of treated cells was added to the slide, cover glasses were put in place immediately and the slides were kept at 4°C. After solidification, the cover glasses were removed and the slides were immersed in pre-chilled lysing solution containing 2.5 mM NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, pH 10, 1% DMSO and 1% Triton X. They were kept for 1 h at 4°C. After lysis, slides were drained completely 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 25 V. After electrophoresis the slides were washed gently with 0.4 mM Tris-HCl buffer, pH 7.4, to remove alkali, after which silver staining was carried out.[13,14] The comets were visualized using an Olympus Magnus research microscope (Olympus India Pvt Ltd, New Delhi, India) and the images captured were analysed using 'CASP' software, which gives the percentage of DNA in tail, tail length, tail moment and Olive tail moment directly.[15]

Measurement of lipid peroxidation

The major damage to membranes in cells and tissues due to γ-irradiation is peroxidation of membrane lipids.[16] Excised tissues such as liver, brain, kidney, spleen and intestinal mucosa were washed out and 10% homogenates were prepared. The lipid peroxidation was measured in terms of thiobarbituric acid reacting substances (TBARS) at 532 nm. The values are expressed as nmoles of malondialdehyde (MDA) per mg protein.[17]

Effect of *Centella asiatica* extract on the survival of mice exposed to a whole-body lethal dose of γ-radiation

Swiss albino male mice (20–25 g body weight) were divided into four groups, each group comprising 10 animals. Animals in Groups I and II were orally administered with distilled water (0.2 ml) and those in Groups III and IV were orally administered with CAE (200 mg/kg body weight in 0.2 ml distilled water). After 1 h of oral administration, Groups II and IV were exposed to 10 Gy whole-body γ-radiation. After radiation exposure, Groups I and II were orally given 0.2 ml distilled water and Groups III and IV were orally given CAE for 7 consecutive days. The percentage survival in each group was recorded.

Statistical analysis

Statistical analyses of the results were performed using ANOVA with Tukey–Kramer multiple comparisons.

Results

Effect of *Centella asiatica* extract against γ-radiation induced strand breaks in plasmid pBR322 DNA

Exposure to γ-radiation led to DNA strand breaks, resulting in the relaxation of plasmid DNA from supercoiled (ccc) form to open circle (oc) form. It was seen that the presence of the extract reduced the radiation-induced disappearance of the ccc form of the plasmid DNA significantly. Figures 2, 3 and 4 demonstrate the effect of different concentrations of CAE on strand breaks in plasmid pBR322 DNA induced by γ-radiation at 25 Gy. It can be seen in Figures 2 and 3 that the presence of CAE (5 mg/ml) during radiation along with the plasmid DNA significantly inhibited the radiation-induced disappearance of the ccc form of plasmid DNA. From the ratios of the slopes of the survival curves for the supercoiled plasmid DNA (Figure 4), the dose-modifying factor was calculated to be 1.92. This indicates that CAE could offer protection against γ-radiation to DNA under in-vitro conditions.

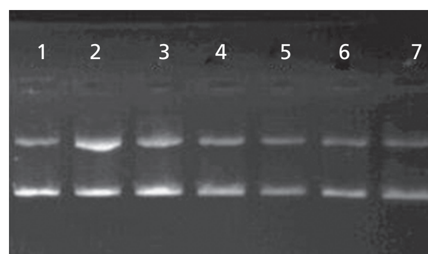

Figure 2 Effect of *Centella asiatica* extract on plasmid pBR322 DNA exposed to γ-radiation. 1: 0 Gy control. 2: 25 Gy control, 3: 25 Gy, 10 mg/ml *Centella asiatica* extract (CAE). 4: 25 Gy, 6 mg/ml CAE. 5: 25 Gy, 4 mg/ml CAE. 6: 25 Gy, 2 mg/ml CAE. 7: 0 Gy, 10 mg/ml CAE.

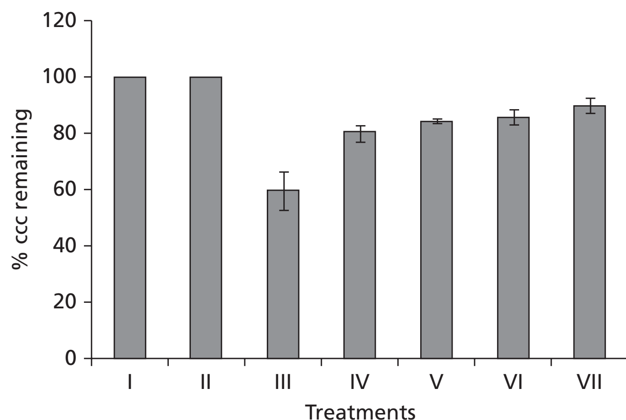

Figure 3 Effect of different concentrations of *Centella asiatica* extract on DNA exposed to γ-radiation. I: 0 Gy, no *Centella asiatica* extract (CAE). II: 0 Gy, 10 mg/ml CAE. III: 25 Gy, no CAE. IV: 25 Gy, 2 mg/ml CAE. V: 25 Gy, 4 mg/ml CAE. VI: 25 Gy, 6 mg/ml CAE. VII: 25 Gy, 10 mg/ml CAE. ccc, supercoiled DNA.

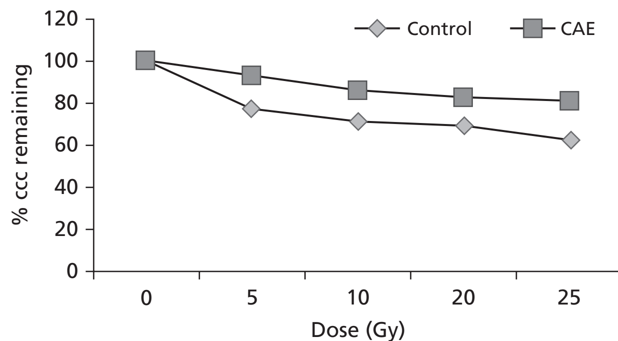

Figure 4 Effect of different dose of γ-radiation in presence of *Centella asiatica* extract on plasmid DNA. CAE, *Centella asiatica* extract; ccc, supercoiled DNA.

Estimation of DNA damage in the tissues of whole-body irradiated mice

The damage to cellular DNA in the bone marrow cells of mice exposed to 4 Gy γ-radiation following oral administration of CAE was studied by alkaline single-cell gel electrophoresis (comet assay) and the results are presented in Figure 5. All the comet parameters (percentage of DNA in tail, tail length, tail moment and Olive tail moment) were found to be elevated in those animals subjected to radiation exposure compared to unirradiated controls, implying the formation of DNA strand breaks due to radiation exposure. When animals were exposed to γ-radiation, tail moment increased to 1.026 ± 0.172 from 0.1065 ± 0.044, % DNA in tail increased to 7.75 ± 0.88 from 1.18 ± 0.237, tail length increased to 5.00 ± 0.787 from 3.045 ± 0.273 and Olive tail moment increased to 1.601 ± 0.0653 from 0.407 ± 0.072. The administration of CAE brought down these parameters to 0.504 ± 0.121, 4.427 ± 0.698, 3.956 ± 0.845 and 0.4199 ± 0.070, respectively, in the bone marrow cells of the irradiated group. This suggests that the administration of CAE significantly inhibits the increase in comet parameters of bone marrow cells in whole-body irradiated animals ($P < 0.001$), indicating that the extract has a protective effect on radiation-induced DNA damage *in vivo* under experimental conditions.

Effect of *Centella asiatica* on lipid peroxidation in different tissues after exposure to 4 Gy γ-radiation

In addition to DNA, membrane constitutes another major vital target for radiation inactivation in living cells. Peroxidation of membrane lipids is one of the major forms of radiation-induced damage in cells, and this peroxidative damage was monitored using the formation of MDA, which reacts with thiobarbituric acid forming a chromogenic substance. Ionizing radiation-induced peroxidative damage to lipids in the membrane causes structural alterations, leading to loss of several vital cellular functions. Whole-body exposure to γ-radiation resulted in increased levels of lipid peroxidation in several tissues of the animals, as can be evidenced from the data presented in Table 1. Administration of CAE to the animals prior to radiation exposure reduced the peroxidation of lipids significantly ($P < 0.001$) in tissues such as liver, brain, kidney and GI mucosa. It can be seen that the decrease in radiation-induced peroxidative damage caused by CAE administration was more pronounced in the brain tissues, implying that there is a radioprotective effect of CAE in the brain.

Effect of *Centella asiatica* extract on 10 Gy γ-radiation-induced mortality

Figure 6 presents the changes in the survival of animals following exposure to an acute lethal dose of 10 Gy whole-body γ-radiation. There was no lethality in unirradiated Groups I and III. Animals in the irradiated groups (Groups II and IV) started dying from day 5. On day 7, the mortality in Group II was 60%, while it was only 30% in Group IV. On day 10 there was 100% mortality in the control irradiated group (Group II) while the mortality was only 70% in the extract administered group (Group IV). This implies that the administration of CAE provided some radioprotection and survival advantage, even at the lethal dose of 10 Gy.

Discussion

The potential application of radioprotective chemicals in the event of planned exposures or radiation incidents has been investigated from the beginning of the nuclear era. The use of radioprotectors represents an obvious strategy to improve the therapeutic index in radiotherapy. However, ideal synthetic radioprotectors that are also safe are presently not available, hence the search for alternative sources, including bioactive compounds of plant origin, has been an ongoing task worldwide.[18,19] Several compounds have been shown to possess the property of protecting various biological systems from the deleterious effects of ionizing radiation. Many of these compounds are only of academic interest because they possess acute toxicity to mammalian systems.[20]

Antioxidants constitute the foremost defense system that limits the toxicity associated with free radicals. Free radical scavenging enzymes such as superoxide dismutase,

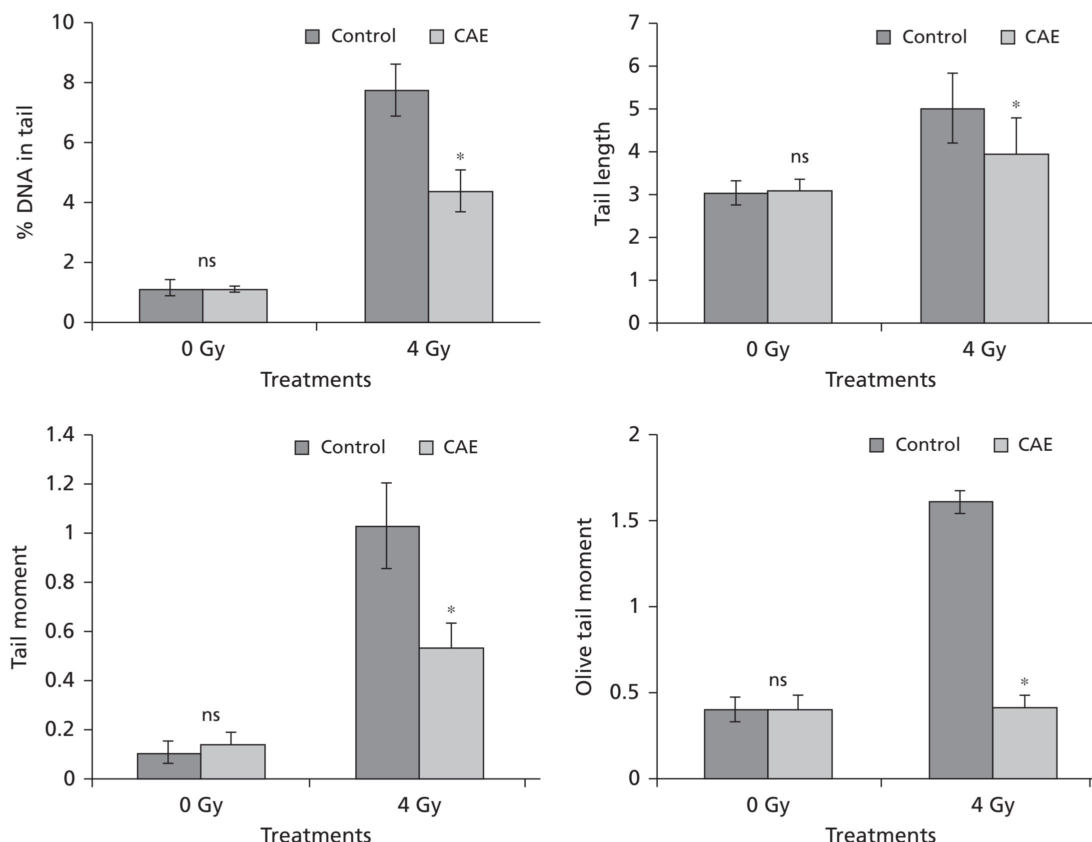

Figure 5 Effect of *Centella asiatica* extract on DNA of mouse bone marrow cells. Comet parameters represent the effect of *Centella asiatica* extract (CAE) administration on γ-radiation (4 Gy) induced DNA strand breaks in the bone marrow cells of whole-body irradiated mice. Mean of the percentage DNA in tail, tail length, tail moment and Olive tail moment are presented as mean ± SD. (ns, not significant; $*P < 0.001$ when compared with respective controls).

glutathione reductase and glutathione peroxidase are the first line of cellular defence against oxidative injury. The equilibrium between these enzymes is an important process for the effective removal of oxygen stress in intracellular organelles.[21] The cellular membrane and DNA are the two main targets of radiation-induced lethality and mutagenicity. The formation of lipid peroxides in tissues exposed to γ-radiation is one of the markers of membrane damage. Per oral administration of the extract protects the animals from radiation-induced lipid peroxidation in different tissues. Ionizing radiations are known to enhance the production of reactive oxygen species that subsequently cause damage to target molecules.[22,23] Superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase are endogeneous antioxidant enzymes that protect cells from oxidative stress. We have already shown that CAE has antioxidant properties, as its presence inhibited the radiation-induced decline in the antioxidant enzymes in the whole-body irradiated (4 Gy)

Table 1 Effect of *Centella asiatica* extract on the peroxidation of lipids in different tissues of mice

Treatment	Liver (nmol/mg)	Brain (nmol/mg)	Kidney (nmol/mg)	Spleen (nmol/mg)	GI mucosa (nmol/mg)
Group I Unirradiated control (0 Gy, DDW)	1.62 ± 0.142	2.64 ± 0.19	0.99 ± 0.13	1.19 ± 0.28	1.38 ± 0.18^{ns}
Group III *Centella asiatica* (200 mg/kg body weight)	1.58 ± 0.11^{ns}	2.32 ± 0.141^{ns}	1.01 ± 0.09^{ns}	0.97 ± 0.008^{ns}	1.36 ± 0.21
Group II Irradiated control (4 Gy, DDW)	3.12 ± 0.28	4.97 ± 0.33	1.91 ± 0.11	1.85 ± 0.17	3.07 ± 0.26
Group IV *Centella asiatica* (200 mg/kg body weight) + 4 Gy	2.11 ± 0.315*	1.65 ± 0.173*	1.24 ± 0.176*	1.33 ± 0.162*	1.53 ± 0.146*

Changes in the lipid peroxidation levels are expressed as nmol malondialdehyde/mg protein. DDW, double distilled water. ns, $P > 0.05$; $*P < 0.001$ when compared with respective control.

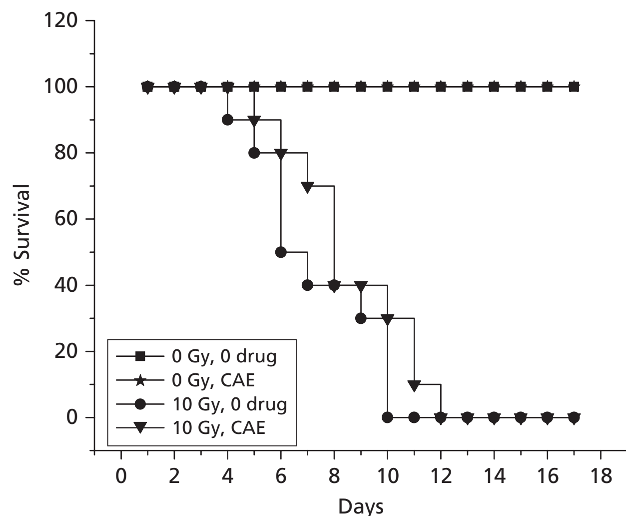

Figure 6 Effect of *Centella asiatica* extract on 10 Gy γ-radiation-induced mortality. CAE, *Centella asiatica* extract.

animals.[24] The elevation of glutathione levels indicates enhanced neutralization of radiation-induced free radicals. This is also supported by the increase in the activity of glutathione-dependent enzymes such as glutathione peroxidase. A relationship between antioxidant properties and radiation protection has been suggested by Shimoi *et al.*[25]

CAE offered protection to DNA from radiation-induced damage. CAE protected pBR322 DNA from γ-radiation-induced strand breaks under in-vivo conditions of radiation exposure. The comet assay has been utilized as a sensitive, rapid and simple technique for the evaluation of DNA damage and repair. Whole-body exposure of mice to a sublethal dose of 4 Gy resulted in damage to the cellular genomic DNA in bone marrow cells, as manifested in the studies on alkaline comet assays. The comet parameters such as tail length, percentage DNA in tail, tail moment and Olive tail moment of the bone marrow cells were found to be increased by radiation exposure. Administration of CAE resulted in inhibition of the increase in the radiation-induced comet parameters, indicating prevention of radiation-induced damage to DNA. Hence the present results clearly show that CAE protected cellular DNA under in-vivo conditions. Thus CAE could be used as a protective agent against ionizing radiation in the mammalian system. As radiation-induced mutations or cancer could originate from lesions in DNA, protection of DNA from these could be an effective means of preventing radiation-induced mutagenesis and carcinogenesis. Thus the present study also implies the use of CAE for preventing radiation-induced cancers.

Mortality of animals following radiation results from various syndromes, depending on the dose. At higher doses, i.e. above 6 Gy, irreversible manifestations of CNS syndrome is the leading cause of mortality. In the present study, after a lethal acute dose of 10 Gy radiation, mortality was 100% on day 10. However, in the CAE-administered group on day 10, the post-radiation survival rate was 30% and 100% mortality was observed only on day 12. On day 6 following irradiation, there was only 50% survival in irradiated Group II while there was 80% survival in the CAE-administered group (Group IV). Thus the results indicate that administration of CAE could enhance the survival of radiation-exposed (10 Gy) animals to a certain extent. Although no radiation survivors were seen on day 12 following the lethal dose of 10 Gy γ-radiation, the group of animals administered with CAE showed a positive survival advantage over the group of animals that was exposed to radiation alone.

Several compounds and plant extracts with antioxidant activity have been reported to be effective radioprotectors.[19,20] Four related triterpenoids (asiatic acid, madecassic acid, asiaticoside and madecassoside) have been found to be the major constituents of CAE.[8] CAE has been reported to have efficient antioxidant activity.[24] The radioprotecting properties of CAE can be attributed to its antioxidant activity.

Conclusions

Most of the biological effects of *Centella asiatica* have been attributed to the presence of ingredients such as triterpenes and phenolic compounds. The present study reveals that *Centella asiatica* extract offers significant protection against DNA damage caused by ionizing radiation, under both in-vitro and in-vivo conditions. CAE provides a survival advantage to animals exposed to a lethal dose of γ-radiation. It has also shown a radioprotective effect against peroxidative damage in membrane lipids of various tissues, including the brain, under in-vivo conditions. The results are suggestive of its application as a radioprotector in humans.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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