Antioxidant, anticlastogenic and radioprotective effect of Coleus aromaticus on Chinese hamster fibroblast cells (V79) exposed to gamma radiation

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Coleus aromaticus (Benth, Family: Lamiaceae), Indian Oregano native to India and Mediterranean, is well known for its medicinal properties. A preliminary study was undertaken to elucidate in vitro free radical scavenging potential and inhibition of lipid peroxidation by C.aromaticus hydroalcoholic extract (CAE). Anticlastogenic and radioprotective potential of CAE were studied using micronucleus assay after irradiating Chinese hamster fibroblast (V79) cells. CAE at 10, 20, 40, 60, 80, 100 and 120 µg/ml resulted in a dose-dependent increase in radical scavenging ability against various free radicals viz., 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azinobis(3-ethylbenzothiazoline–6–sulfonic acid) (ABTS), superoxide anion (O2•-), hydroxyl (OH•) and nitric oxide (NO•) generated in vitro. A maximum scavenging potential was noticed at 100 µg/ml and a saturation point was reached thereafter with the increasing doses of CAE. The free radical scavenging potential of the extract was in the order of DPPH > ABTS > Superoxide > Hydroxyl > Nitric oxide. CAE also exhibited a moderate inhibition of lipid peroxidation in vitro, with a maximum inhibition at 60 µg/ml (33%), attaining saturation at higher doses. The extract also rendered protection against radiation induced DNA damage, as evidenced by the significant (P < 0.05) decrease in the percentage of radiation-induced micronucleated cells (MN) and frequency of micronuclei (total). A maximum anticlastogenic effect/ radioprotection was noticed at a very low concentration i.e., 5 µg/ml of CAE, treated 1 h prior to 2 Gy of gamma radiation. A significant (P < 0.0001) anticlastogenic/radioprotective effect was also observed when the cells were treated with an optimum dose of CAE (5 µg/ml) 1 h prior to 0.5, 1, 2 and 4 Gy of gamma radiation compared with the respective radiation control groups. Overall, our results established an efficient antioxidant, anticlastogenic and radioprotective potential of CAE, which may be of great pharmacological importance.

Introduction

Ionizing radiation generates reactive oxygen species such as superoxide anion (O2•-), the hydroxyl radical (OH•), singlet oxygen (O2), nitric oxide (NO), hydrogen peroxide (H2O2) and peroxyl radicals in a biological system, by radiolysis of water that can damage several cellular components and bio-molecules such as DNA, proteins, lipids, amino acids and carbohydrates (1–3). Many disease states in the human body such as aging, arthritis, coronary disease, Alzheimer’s disease, cataract and cancer are known to be free-radical mediated and can be counteracted by the use of antioxidants (4). These antioxidants work by either stopping free radical damage by donating an electron without becoming a damaging free radical themselves, or by preventing oxidation of critical cellular bio-molecules.

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. DNA damage occurs either by direct ionization or indirectly through generation of free radicals that attack DNA, resulting in single-strand breaks and oxidative damage to sugar and base residues (2,5) which may later be converted into DNA double strand breaks (DSBs). It is understood that the unrepaird DSB will contribute to chromosomal aberrations. aberrations like fragments, dicentrics and chromosomes with damaged kinetocore following division appear as micronuclei in the daughter cells (6). The micronucleus count gives an indirect measure of cytogenetic damage induced by any genotoxic agent and the inhibition of radiation-induced micronuclei indicates the radioprotective potential of any test agent. Recently, several of such phytochemicals viz., carotenoid (beta-carotene), curcumin, ascorbic acid and flavonoids (orientin and vicinin) have demonstrated anti-genotoxic potential along with a significant antioxidant activity (7–9). Since radiation-induced genotoxicity is predominantly a free-radical mediated effect on DNA, it may be logical to expect that the plant-derived bioactive compounds with their antioxidant potential may also render radioprotection to normal tissues as evidenced from our earlier experience (8–10).

The uses of medicinal plants in traditional medicine is widespread and still serve as leads for the development of novel pharmacological agents. Many such medicinal plants have hepatoprotective, neuroprotective, anti-inflammatory and also antioxidant or radical-scavenging properties (11,12). Therefore, in recent years the field of clinical medicine, major emphasis is being placed on the use of antioxidants mainly for intervening and sometimes for treating several human ailments. Coleus aromaticus (Benth, Family: Lamiaceae), is one such Indian medicinal plant used extensively to treat a spectrum of ailments. It is a small herb, 30–90 cms in height with fleshy leaves and cultivated throughout India. It is used by almost all local people for its therapeutic efficacy against common cold, cough, fever, headache and indigestion. The leaves are said to have specific action on the bladder and to be very useful in urinary disease and vaginal discharges (13). Therefore, the present study was conducted to elucidate in vitro antioxidant, anticlastogenic or radioprotective potential of the

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hydroalcoholic extract of Caromatus using micronucleus assay as an experimental endpoint.

Materials and methods

The chemicals, 2, 2-diphenyl-2-picryl hydrazyl (DPPH), 2, 2-azinobis (3-ethylbenzothiazoline–6-sulfonic acid; i.e. ABTS), Ethylenediaminetetraacetic acid (EDTA), Cytochalasin-B, Thioarbituric acid (TBA), Fetal calf serum (FCS), Dulbecco’s Minimal Essential Medium (DMEM) and L-glutamine were purchased from Sigma Chemical Co. (St. Louis, USA). Acridine orange was procured from Gurr (BDH, UK), Butylated hydroxytoluene (BHT) from Merck (Germany) and all the other standard laboratory chemicals were obtained from Qualigens Fine Chemicals, India.

Preparation of hydroalcoholic extract of Caromatus (CAE)

The plant Caromatus was identified by Prof. Rajgopal,P.K., Taxonomist, Department of Botany, Mahatma Gandhi Memorial College, Udupi, India. The leaves from the plants were collected during the months of April and May of the year, cleaned with water, shade-dried and powdered. The hydroalcoholic extract was prepared by the extraction of 100 g of leaf powder in 50% of methanol in water (total volume 50 ml) at 50°C in a Soxhlet apparatus for 72 h. (14). The liquid extract was then cooled and concentrated by evaporation. The extract was stored at −20°C until use.

Radical generation and scavenging studies

A stock solution of 1 mg/ml of CAE was prepared in deionized water and diluted to get various concentrations (10–120 μg/ml) in a final volume of the reaction mixture. The free-radical scavenging activity of the extract was analysed by following the various standard in vitro radical generating model systems viz., 1.1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethylbenzothiazoline–6-sulfonic acid) (ABTS), superoxide anion (O2⁻), hydroxyl (OH) and nitric oxide (NO⁻). In all the experiments, deionized water served as blank and reaction mixtures without CAE served as control samples. The changes in the absorbance of the reaction mixtures were measured using a spectrophotometer (VIS-260, Shimadzu Corp., Japan) and the percent scavenging or inhibition was calculated according to the following formula.

\[
\text{Percent scavenging or inhibition} = \left(\frac{\text{Absorbance of control} - \text{Absorbance of test/absorbance of control}}{\text{Absorbance of control}}\right) \times 100
\]

DPPH scavenging activity of CAE in vitro

The effect of CAE on the DPPH radical was estimated according to the method of Hou et al. (15). The principle of the reduction of DPPH free-radical assay is that antioxidants react with the stable DPPH radical and convert it into 1,1-diphenyl-2-picrylhydrazine. The ability to scavenge the stable DPPH radical is measured by a decrease in the absorbance. Aliquots containing various concentrations (10–120 μg) of CAE in the final volume of 2 ml were mixed with 2 ml of 0.05 mM DPPH (in methanol). DPPH (2 ml) with equal amount of PBS served as control. Reaction mixtures were incubated at 37°C for 20 min and the absorbance of reaction mixtures was recorded at 517 nm.

ABTS radical decolourization assay

ABTS diammonium salt radical cation decolourization test was performed using spectrophotometric method of Pelligrini et al. (16). The principle of the ABTS (2,2-azinobis (3-ethylbenzothiazoline–6-sulfonicacid) diammonium salt cation radical decolorisation assay is that the antioxidants react with ABTS resulting in the decolorisation of the ABTS radical in aqueous phase. The ABTS stock reagent mixture was prepared by mixing 88 μl of 140 mM potassium persulfate (K2S2O8) with 5ml of 7 mM of ABTS stock solution (pH 7.4). The working ABTS reagent was prepared by diluting the stock solution with ethanol to give an absorbance of 0.7 ± 0.5 at 734 nm. Various concentrations of CAE (10–120 μg) in a final volume of 1 ml of PBS were mixed with 1 ml of the ABTS cation working solution. The ABTS working solution with equal volume of PBS served as control. The reaction mixtures were incubated at room temperature (28°C) for 30 min and the absorbance was measured at 734 nm.

Hydroxyl radical scavenging activity of CAE

Hydroxyl radical scavenging assay was performed by the oxidation of deoxyribose using Fe²⁺–EDTA–ascorbate–H₂O₂ system according to the method of Halliwell et al. (17). The degradation of deoxyribose by hydroxyl radicals was estimated by measuring the TBA reactive substances (TBARs). The reaction mixture in a final volume of 1 ml contained ascorbic acid (50 mM), EDTA (2 mM), FeCl₃ (20 μM), H₂O₂ (1.42 mM), deoxyribose (2.8 mM) and various concentrations of CAE (10–120 μg). The reaction mixture without CAE served as control. The reaction mixtures were incubated at 37°C for 30 min, followed by the addition of 1ml of 5% trichloroacetic acid (TCA) and 1 ml of TBA and heated in a waterbath maintained at 75°C for 30 min and cooled. The absorbance was measured at 534 nm using a spectrophotometer.

Scavenging of nitric oxide by CAE

The nitric oxide scavenging potential of CAE was determined according to the method of Sreekayyan and Rao (18), with minor modifications. Briefly, 1 ml of sodium nitroprusside (5 μM) in PBS containing various concentrations of CAE (10–120 μg) along with the control (sodium nitroprusside without CAE) were incubated at room temperature (28°C) for 2.5 h, followed by the addition of 1 ml of Greiss reagent (prepared by mixing an equal volume of 1% sulfanilamide in 2% phosphoric acid with 0.1 % N- (naphthyl) ethylenediamine hydrochloride in water). The absorbance of the chromophores formed during diazotization of nitrate with sulfanilamide and the subsequent coupling with N-(1-naphthyl) ethylenediamine was read at 546 nm.

Superoxide radical scavenging activity of CAE

Superoxide scavenging activity of CAE was performed by photo-oxidation of riboavblin according to the method of McCord and Fridovich (19). The reaction mixture in a final volume of 3 ml containing 20 mM phosphate buffer, 100 mM EDTA-Sodium cyanide, 2 mM of riboflavin, 75 mM NBT and various concentrations of CAE (10–120 μg) along with the control (reaction mixture without CAE) were illuminated under incandescent light for 20 min and the absorbance was recorded at 513 nm.

Inhibition of lipid peroxidation by CAE

Lipid peroxidation was carried out according to the method of Shoji et al. (20) with minor modifications. Briefly, 10% (w/v) mouse brain homogenate was prepared in 150 mM potassium chloride. The reaction mixture contained 300 μl of brain homogenate, 100 μl of 150 mM potassium chloride and various concentrations of CAE (10–120 μg) in a final volume of 1 ml. The reaction mixtures without CAE served as control. Peroxidation was initiated by adding 10 μl of 20 mM ferrous sulfate (FeSO₄). After incubating the mixture for 20 min at 37°C, the reaction was stopped by the addition of 1 ml of ice-cold solution of 0.25N HCl containing 15 % TCA, 0.38% TBA and 0.05% BHT and heated in a water bath at 65°C for 30min. The reaction mixtures were cooled, centrifuged at 10000 rpm, for 10 min; absorbance of the TBARS in the supernatant was recorded at 532 nm.

Cell line and culture

Chinese hamster lung fibroblast (V79) cells procured from the National Center for Cell Sciences, Pune, India, were maintained in our laboratory and used in the present study. The cells were cultured in DMEM supplemented with 10% FCS, L-glutamine (2 mM), 100 U/ml of penicillin and 100 μg/ml of streptomycin. Cells were routinely cultured in 25 cm² flasks (Nunc, Roskilde, Denmark) with loosened caps and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

Experimental design

Influence of CAE on radiation-induced cytogenetic damage. The experiment was carried out with V79 cells that were trypsinized and an appropriate number (5 × 10³ / fl culture flask) of cells were seeded into 25 cm² T-flasks in triplicate for each treatment group and allowed to grow. Once they attained exponential phase (i.e. 24 h after seeding), the culture flasks were divided into different groups for various treatments. The first triplicate set of the culture flasks served as sham treated control (without any treatment). In the second group, cells were treated with CAE at a dose of 5 and 1000 μg for 1 h without irradiation. In the third group, cells were treated with increasing doses of CAE (i.e. 10, 50, 0, 10, 20, 50, 100, 500 and 1000 μg/ml), 1 h before 2 Gy of gamma radiation. The cells in the fourth and fifth groups were exposed to various doses of gamma radiation (0.5, 1, 2 and 4 Gy) with or without exposure of the selected optimum dose of CAE (5 μg/ml) for 1 h prior to the radiation treatment.

Treatment of the cells with CAE and irradiation procedure

The CAE was dissolved in DMEM and sterilized by passing through a 0.22 μm syringe filter (Millipore). Since the stability of the various constituents of CAE was not evaluated at present, as a precautionary measure, CAE was prepared freshly each time just before the treatment. V79 cells were treated with various concentrations of CAE (1.0–1000 μg/ml) for 1 h and the drug treatment was terminated by gently washing with culture media followed by the addition of 5 ml of fresh growth medium. The cells were exposed to gamma radiation at a dose rate of 1.0 Gy/min with a source-to-surface distance of 87.5 cm from a Cobalt-60 teletherapy unit (Theratron 780-C, Atomic Energy Agency, Ontario, Canada) of Shirdi Sai Baba Cancer Research Center, Kasturba Medical College Hospital, Manipal.
**Micronuclei in vitro assay.** Immediately after the irradiation of V79 cells, 4 µg/ml of cytochalasin-B (stock dissolved in DMSO and diluted in media before use) was added to each culture flask to block cytokinesis and cultures were incubated further for 16 h. The cells were detached from the flask by trypsin treatment (0.1%) for 2 to 3 min with subsequent inactivation of the trypsin action by the addition of 1 ml of DMEM containing serum. The single-cell suspensions thus prepared were centrifuged (1000 rpm for 10 min), washed with PBS and subjected to a mild hypotonic (0.75% KC1) treatment for 1 min. The cells were then centrifuged (1000 r.p.m. for 10 min) and fixed in Carnoy’s fixative (3:1 methanol : acetic acid) for 30 min. Finally, the cells were resuspended in a small volume of (100–200 µl) of fixative and dropped onto precleaned slides and air-dried. The slides were coded to avoid observer’s bias and stained with 0.002% acridine orange in Sorensen’s buffer (pH 6.8) for several seconds. Slides were covered with a coverslip and observed under a fluorescent microscope (Photomicroscope III, Carl Zeiss, Germany) using a 40X neofluar objective. A minimum of 1000 binucleated cells with well-preserved cytoplasm were scored from each group for the presence of one/two or three micronuclei. The micronuclei were identified and scored by a single person according to the criteria of Fenech and co-workers (21) and expressed as percent micronucleated binucleate cells (MNBNC %) with one/two two or three micronuclei in each group.

**Statistical analysis**

All the data were expressed as Mean ± SEM. The dose response curve for MN was fitted on a linear model (Y = α + βX). The MNBNC% and total MN% or frequency of micronuclei (total number of micronuclei/number of cells counted × 100) in different treatment groups was compared with the control by a One-way ANOVA test and comparison between the radiation alone and the optimum dose of CAE by a Student’s ‘t’ test using GraphPAD InStat, Software, USA.

**Results**

**Scavenging of stable DPPH and ABTS free radicals by CAE** (Figure 1)

The DPPH radical is widely used as a model system to investigate the free-radical scavenging activities of several plant extracts. Figure 1 shows the dose-response curve for CAE on DPPH radical scavenging activity. CAE scavenged the DPPH radicals in a concentration-dependent manner with the maximum scavenging activity of 80% at 80 µg/ml attaining saturation with further increase in the CAE concentration. Similarly CAE demonstrated ABTS radical scavenging activity in a dose-dependent manner with 74.25% at 80 µg/ml CAE concentration attaining saturation with higher concentrations (Figure 1).

**Nitric oxide, superoxide radical and hydroxyl radical scavenging activity of CAE**

Figure 1 also show the potential of CAE in scavenging nitric oxide, superoxide radical and hydroxyl radical in a concentration-dependent manner (10–120 µg/ml) with the saturation point reaching a concentration of 80 µg/ml for NO and 100 µg/ml for superoxide and hydroxyl radical.

**The effect of CAE on lipid peroxidation**

The addition of CAE to the reaction mixture resulted in a moderate dose-dependent inhibition of lipid peroxidation with the maximum inhibition of 35% at 60 µg/ml and saturated thereafter (Figure 2).

**Protection of radiation-induced cytogenetic damage by CAE**

The CAE by itself did not produce any genotoxic or clastogenic effect on V79 cells at 5 µg/ml and even at the highest dose (1000 µg/ml) used in the present study as indicated by the comparable MN counts with that of sham treated control V79 cells (Table I and Figure 3). Two Gy of gamma radiation, which produced 16.47% of micronucleated cells and 17.87% of total micronuclei, was selected to study the influence of various concentrations of CAE on the radiation-induced cytogenetic damage (Table I and Figure 3). Treating the V79 cells with various doses of CAE (1, 5, 10, 20, 50, 100, 500 and 1000 µg/ml) for 1 h before 2 Gy of gamma radiation resulted in a significant decrease in the percentage of micronucleated cells and total micronuclei when compared with the radiation alone group. The maximum reduction (51%) in the radiation-induced micronuclei induction was observed when 5 µg/ml of CAE was administered before 2 Gy of gamma radiation. However, further increase in the CAE did not produce significant enhancement in the protection. Treating the cells for 1 h with the highest dose of CAE, i.e. 1000 µg/ml, before 2 Gy produced only 23% protection, although...
MNBNC% and the total micronuclei remained significantly lower when compared with 2 Gy alone.

The optimum radioprotective dose, 5 µg/ml of CAE that produced maximum protection, was selected for radiation dose response studies. The V79 cells exposed to different doses of radiation induced a dose-dependent linear increase in the MN counts ($R = 0.9980$, Table II and Figure 4). The lowest radiation dose, 0.5 Gy, produced only 5.37% micronucleated cells and 5.73% of total micronuclei, while the higher dose of radiation (4.0 Gy) produced 33.87% micronucleated cells and 39% of total micronuclei. Pretreatment of V79 cells for 1 h with an optimum dose of CAE (5 µg/ml), before exposure to increasing doses of gamma radiation, i.e. 0.5, 1.0, 2.0 and 4.0 Gy, produced a significant ($P < 0.0001$) decrease in both percentage of micronucleated cells as well as total micronuclei compared with the respective radiation alone groups (Table II and Figure 4). The reduction in the frequency of percent MNBC and percent total micronuclei was by a factor of $>1.6$ when compared with the respective radiation alone groups. Although the frequency of one, two and multiple micronuclei was scored separately, the frequency of multinucleated cells was observed only at the higher dose of radiation (4 Gy) (Table II). Pretreatment of V79 cells for 1 h with 5 µg/ml of CAE before 2 and 4 Gy gamma radiation.

**Table I.** Effect of various concentrations of CAE on radiation induced micronuclei in V79 cells

<table>
<thead>
<tr>
<th>CAE (µg/ml) + RT</th>
<th>Total MNBNC (%) (Mean ± SEM)</th>
<th>Total MN (%) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control/sham</td>
<td>1.07 ± 0.18</td>
<td>1.07 ± 0.18</td>
</tr>
<tr>
<td>5.0 + 0.0 Gy</td>
<td>0.83 ± 0.15$^a$</td>
<td>0.83 ± 0.15$^a$</td>
</tr>
<tr>
<td>1000 + 0.0 Gy</td>
<td>0.97 ± 0.07$^a$</td>
<td>0.97 ± 0.07$^a$</td>
</tr>
<tr>
<td>2.0 Gy</td>
<td>16.47 ± 0.41$^b$</td>
<td>17.87 ± 0.55$^b$</td>
</tr>
<tr>
<td>1.0 + 2.0 Gy</td>
<td>10.70 ± 0.21$^c$</td>
<td>11.17 ± 0.35$^c$</td>
</tr>
<tr>
<td>5.0 + 2.0 Gy</td>
<td>8.03 ± 0.18$^d$</td>
<td>8.40 ± 0.17$^d$</td>
</tr>
<tr>
<td>10 + 2.0 Gy</td>
<td>8.63 ± 0.15$^e$</td>
<td>8.97 ± 0.23$^e$</td>
</tr>
<tr>
<td>20 + 2.0 Gy</td>
<td>10.57 ± 0.26$^c$</td>
<td>10.87 ± 0.38$^c$</td>
</tr>
<tr>
<td>50 + 2.0 Gy</td>
<td>10.90 ± 0.44$^d$</td>
<td>11.27 ± 0.50$^d$</td>
</tr>
<tr>
<td>100 + 2.0 Gy</td>
<td>11.27 ± 0.47$^d$</td>
<td>11.73 ± 0.58$^d$</td>
</tr>
<tr>
<td>500 + 2.0 Gy</td>
<td>12.72 ± 0.23$^e$</td>
<td>12.67 ± 0.33$^e$</td>
</tr>
<tr>
<td>1000 + 2.0 Gy</td>
<td>12.70 ± 0.44$^f$</td>
<td>13.33 ± 0.63$^f$</td>
</tr>
</tbody>
</table>

CAE: Coleus aromaticus extract; RT: Radiation treatment; MNBNC: Micronucleated binucleate cells; Total MN%: Total micronuclei (single, two and three or more) in 1000 binucleated cells; SEM: Standard Error of the Mean.

$^a$Non significant.

$^b$P < 0.001 versus control/sham.

$^c$P < 0.01.

$^d$P < 0.05 versus RT (2 Gy) alone.

**Table II.** Effect of optimum dose of CAE (5 µg/ml) on radiation induced micronuclei

<table>
<thead>
<tr>
<th>RT (Gy)</th>
<th>Radiation alone group</th>
<th>CAE (5 µg/ml) + RT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total MNBNC (%) (Mean ± SEM)</td>
<td>Total MN (%) (Mean ± SEM)</td>
</tr>
<tr>
<td>0</td>
<td>1.00 ± 0.17</td>
<td>1.03 ± 0.15</td>
</tr>
<tr>
<td>0.5</td>
<td>5.37 ± 0.22</td>
<td>5.73 ± 0.20</td>
</tr>
<tr>
<td>1</td>
<td>9.63 ± 0.15</td>
<td>10.17 ± 0.23</td>
</tr>
<tr>
<td>2</td>
<td>16.53 ± 0.38</td>
<td>17.70 ± 0.50</td>
</tr>
<tr>
<td>4</td>
<td>33.87 ± 0.67</td>
<td>39.00 ± 0.90</td>
</tr>
</tbody>
</table>

$^a$P < 0.00001 versus RT alone; No symbol: Non-significant. Other details as in Table I.
resulted in a significant \( (P < 0.05-0.001) \) decline in the cells containing two or three micronuclei when compared with the respective radiation alone groups (Table II).

**Discussion**

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 principles of plant origin, has been an ongoing task worldwide. The present work demonstrated the phytotherapeutical significance of *C. aromaticus*, a popular medicinal plant widely used in the Indian system of medicine, Ayurveda. Several earlier studies suggested that medicinal plants, their phytotherapeutical preparations or isolated constituents as supplements of human diets were promising preventative agents for the pathogenesis of a spectrum of diseases (4,7,11) and also rendered radioprotection (22). The literature on *C. aromaticus* and its related species for specific medicinal properties is scarce. To the best of our knowledge this is the first scientific report on the radioprotective potential of CAE.

The results of the *in vitro* antioxidant activity data showed a significant free-radical scavenging effect of CAE on DPPH, ABTS, nitric oxide, superoxide and hydroxyl radicals in a dose-dependent manner. Such free radical scavengers have a key role in radioprotection, because radiation induced cytotoxicity is mediated mainly through generation of free radicals and their action on DNA within the biological system (2,5). Thus free-radical scavenging appears to be a likely mechanism of protection by CAE for the benefit of radioprotection. Besides DNA, membrane lipid damage is considered a critical factor in radiation-induced cellular damage and reproductive cell death (3,23,24). Moreover, Przybyszewski et al. (25) have reported that the products of lipid peroxidation, such as malonyldialdehyde (MDA), damage the enzyme systems and even the DNA. Therefore, the anti-lipid peroxidation effect of CAE observed in the present study may be of significance for the inhibition of radiation-induced lipid peroxidation.

It is well established that ionizing radiation induces different types of lesions in the DNA, including single and DSBs, base damage as well as DNA cross-links (DNA–DNA and DNA-Protein). Among all these, DNA DSBs have been considered the critical lesion for the radiation-induced chromosome break (26) and cell death (27,28). There was a correlation established between the induction of cell death and chromosome aberrations and the frequency of micronuclei induction (29). These micronuclei are formed from acentric fragment(s) or sometimes from the whole chromosome (with defective kinetocore) (6). The cytogenetic damage induced by radiation and clastogenic agents on the mitotic cell is expressed as an increase in the micronuclei frequency during the interphase after the first post-treatment mitosis. Moreover, it is understood that non-repair/misrepair of the DNA double-strand breaks contributes to the chromosomal aberrations (26,30) which could be analysed by the quantitative analysis of micronuclei. Therefore, the micronucleus assay is a very useful parameter used for assessing cytogenetic damage and is extensively used to screen the cytotoxic/radiomodifying potential of synthetic and natural products (9,31). Our studies demonstrated that CAE was effective in counteracting the clastogenic effect of radiation and thereby offering radioprotection as assessed by the micronucleus assay. CAE was non-toxic to the V79 cell even at the highest dose (1000 \( \mu \text{g/ml} \)) tested. It is interesting to note that CAE was very effective in ameliorating the effect of radiation even at 1 \( \mu \text{g/ml} \), the lowest dose used. Moreover, a significant protection window was observed for the range of radiation doses. An earlier study on the ethanolic extract of *C. aromaticus* demonstrated the anticlastogenic effect against cyclophosphamide and mitomycin–C induced mouse bone marrow protection (32). Here we report for the first time the radioprotective potential of *C. aromaticus*. Although, at this stage, the exact mechanism of the action of CAE on radioprotection is far from being understood, the available information on the antioxidant potential of CAE obtained from the present investigation supports the free radical scavenging mediated pathways. The earlier report on the chemical constituents of the leaves of *C. aromaticus* indicated the presence of carvacrol, thymol, eugenol, chavicol, ethyl salicylate, chlorophyllin, flavonoids (cirsimaritin) and \( \beta \)-sitosterol-\( \beta \)-D-glucoside (33,34). Therefore, the presence of some of these chemical agents may have rendered a radioprotective effect. Moreover, ascorbic acid, eugenol and flavonoids are known to have antioxidant and radioprotective properties. The other constituents, chlorophyll and chlorophyllin, demonstrated antimutagenic and anticlastogenic effects (35,36); hence, they may also have contributed to the radioprotective effect of CAE observed in the present study. Besides, the plant may also have other unidentified active principles. Therefore, although CAE is capable of imparting a radioprotective effect, at this stage it is not possible to attribute the observed effect to any one of the bioactive principles of the plant as the degree of protection will depend on the effect of these agents either singly or collectively against radiation-induced DNA damage.

In the present study, both the antioxidant and anticlastogenic effect were more pronounced at the lower concentration of CAE as observed by the significant free-radical scavenging property, inhibition of lipid peroxidation and cytogenetic protection. This observation clearly demonstrates the presence of an optimum dose, for the exertion of CAE’s maximum effect. Increasing the dose of CAE above the optimal dose did not impart any desired effect in the micronucleus study. As the active component/s responsible for the observed radioprotective effect/antioxidant potential have not yet been identified, it may be difficult to explain why higher doses of *C. aromaticus*, despite having more enhanced antioxidant properties, did not enhance the anticlastogenic and radioprotective properties as do the lower doses. It could be speculated that the preferential uptake of the active constituents into V79 cells must have elicited a significant radioprotective effect at the lower doses; while at the higher doses the increase in the radioprotective potential was not proportional to the increase in the dose. However, the reduction in the induction of radiation-induced micronuclei was significant even at the highest dose of CAE, 1000 \( \mu \text{g/ml} \), used in this study. Prasad *et al.* (32) observed that the ethanolic extract of *C. aromaticus* was found to be more effective in imparting its radioprotective effect at its lower dose; and beyond this dose resulted only in an additive effect.

It is concluded that the active principles of the CAE need to be isolated, purified and characterized in order to understand the mechanism of its radioprotection. The antioxidant function of CAE in reducing clastogenicity may also be due to the induction of phase II detoxification enzymes such as superoxide dismutase, catalase etc., which needs to be confirmed by *in vivo* studies in future. However, *in vivo* situations may result in different observations because the many enzyme systems,
bioavailability problems, metabolic products etc., make situations different from those in the in vitro situation, and more difficult to understand as the drug in this condition comes into direct contact with the free radicals. Therefore, the radioprotective potential of CAE itself and active principles need to be explored extensively in animal studies and by using human cells. Besides its radioprotective potential, the present observation of the antioxidant potential of the CAE could be of great significance in chemoprevention and may be useful in combating various free-radical mediated human pathological conditions.

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