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Cytogenetic and biochemical competency of chamomile essential oil against γ-rays induced mutagenic effects in mice

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► ***Original article***

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ABSTRACT

Background: Chamomile essential oil (CEO) hauls out from *Matricaria chamomilla L.*, is a well-known anti-oxidant. Oxidative stress induces clastogenic and biochemical disorders after γ-irradiation of animals. ***Materials and Methods***: Mice were divided into five groups. Control group received vehicle only. CEO-treated group received CEO. Irradiated group received vehicle and exposed to γ-rays. Pre-treated group received CEO ½h before γ-rays exposure. Post-treated group received CEO ½ hour after γ-rays exposure. Peripheral-blood micronucleus (PMN), bone-marrow micronucleus (BMN), frequency of chromosomal aberrations (CAs), reduced glutathione (GSH), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (PGx) and myeloperoxidase (MPO), lactate dehydrogenase (LDH) and tumor necrosis factor-α (TNF-α) parameters were assessed. ***Results:*** In irradiated mice group, PMN score, BMN occurrence and CAs were increased when compared with control mice group. In addition, significant increases in levels of liver lipid peroxidation (LP); expressed as MDA and TNF-α. In addition, activities of liver MPO and LDH were found. Besides, significant decreases in content of GSH, activities of SOD and PGx in liver tissues were recognized. CEO treatment (1.0 g/kg body weight) before- and after-irradiation ameliorated all these biochemical indices, as well as cytogenetic alterations induced by γ-rays when compared with irradiated group, indicating that pre- or post-treatment with CEO significantly attenuates the acute hazards caused by γ-rays exposure. ***Conclusion:*** The data suggest that CEO possesses a radioprotective potential against γ-radiation induced cytogenetic and biochemical damages in mice.

Keywords: *Chamomile oil, radioprotector, cytogenetic disorder, biochemical alteration, γ-rays.*

INTRODUCTION

Matricaria chamomilla L., (family *Asteraceae*) commonly known as chamomile is a plant used in traditional medicine for many therapeutic purposes [(1)]. The CEO was found to contain 13 compounds, mainly bisabolol and its oxides, apigenin, chamazulene, farnesene, germacrene and other sesquiterpenes [(2-4)]. Chamomile possesses anti-oxidant, anti-proliferative, anti-inflammatory effects and reduces mutagenicity in mice. It was found that, *in vivo*, CEO induced inhibition of genotoxicity produced by daunorubicin (DAU) in mouse bone marrow cells [(5)] and *in vitro* study, CEO was efficient chemo protective agent against the damage induced by DAU in the precursor cells of the germinal line of mice [(1)].

CEO has a nephroprotective effect in kidney ischemia/ reperfusion related to acute kidney injury essentially through oxidative stress [6]. Until present, there are not fully effective therapeutic agents against Acanthamoeba strains; causative agents of a fatal encephalitis and severe keratitis in humans and other animals, recently Hajaji *et al.* [7] concluded that CEO could be used as a future therapeutic agent against Acanthamoeba infections.

Mice were used as model animals because they have common or similar responses, at least in part, to -irradiation to those seen in human. The young-mouse model could be successfully used for the integration of genotoxicity and toxic kinetic studies in the radiation-risk assessment process due to higher sensitivity in comparison to adult rats [8]. Ionizing radiation-induced oxidative damage in all animal organs, it produces reactive oxygen species (ROS) [9] that lead to diminish of tissue antioxidant defense system [10] and implicates in the process of cellular macromolecules, DNA damages, and mutagenesis [11] hence, it is reasonable to assume that CEO capable of scavenging free radicals and play a significant role in modulating these processes. However, no studies on the chamomile's biochemical competency and anti-mutagenic ability against radiation have been made.

The aim of the present study was to gather further knowledge regarding CEO to determine its radio protector potency to reduce biochemical disorders and/or genotoxicity, in γ-rays exposure animal model.

MATERIALS AND METHODS

Animals

Young mice (21-25 days old, weighing 15±2.5g) were obtained from the Holding Company for Biological Products and Vaccines (Helwan, Cairo, Egypt) and received humane care. This study complies with National Institutes of Health guidelines. The animals are maintained under room temperature (25±2°C), humidity (60±10%) and 13h light & 11h dark cycles. Normal mouse food was supplied and fed to the animals *ad libitum*. Animals were acclimatized to the experimental conditions prior to CEO dosing for 3 days. The study was approved by Central Scientific Publishing Committee, Egyptian Atomic Energy Authority, RF-158, 7-2016.

Chemicals and CEO treatment

Chemicals were purchased from Sigma-Aldrich, USA. Corn oil and CEO; extracted from the flowers of *M. chamomilla*, obtained from Gritman Co., USA. The two oils maintained at 4ºC until use. Corn oil used as a vehicle. Each CEO dose is diluted in 0.4ml of corn oil.

Experimental design

Five groups with six animals each were organized for assay: A control group received vehicle only. CEO-treated group received CEO (1.0g/ kg body weight) orally, according to Hernandez-Ceruelos *et al.* [5]. Irradiated group received 0.4ml of vehicle and exposed to 4Gy γ-rays (performed with ^{137}Cs Gamma Cell-40 at NCRRT, Nasr City, Cairo, Egypt; at a dose rate 0.422Gy/ minute). Pre-treated group received CEO ½h before γ-rays exposure. Post-treated group received CEO ½ hour after γ-rays exposure. Mice were killed by cervical dislocation 24 hours after treatment or exposure.

Liver was excised immediately in cold 0.9% NaCl, then removed and rinsed in chilled 0.15M Tris KCl buffer, pH 7.4 to yield 10% (w/v) homogenate.

Biochemical analysis

Reduced GSH levels were determined in liver tissue by the methods described by Ellman [12], which is based on the reduction of Ellman's reagent [5,5-dithiobis-(2-nitrobenzoic acid)] by SH-groups to form 1mole of 2-nitro-5-mercaptobenzoic acid/mole of SH. The nitro-mercaptobenzoic acid has an intense yellow color and can be determined by spectrophotometer. The MDA level was determined spectrophotometry in liver tissue according to the method of Buege and Aust [13]. 0.5 ml of tissue homogenate was shaken with 2.5 ml of 20% trichloroacetic acid in a centrifuge

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tube. To the mixture, 1 ml of 0.67% thiobarbituric acid was added, shaken and warmed for 30minutes in a boiling water bath followed by rapid cooling. Then 4 ml of n-butyl-alcohol was added and shaken. The mixture was centrifuged at 800×g for 10 minutes. MDA content was determined from the n-butyl-alcohol layer at 535nm absorbance. The results were expressed as nmol/g tissue.

Activity of liver tissue SOD was determined according to the method of Minami and Yoshikawa [14]. The method is based on the generation of superoxide anions by pyrogallol autoxidation, detection of generated superoxide anions by nitro blue tetrazolium formazan color development and measurement of the amount of generated superoxide anions scavenged by SOD (the inhibitory level of formazan color development). The GPx activity in liver tissue was determined according to the method of Lawrence and Burk [15]. This method is based on measuring the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) using hydrogen peroxide as the substrate. A reaction mixture of 1ml contained 50mM potassium phosphate buffer (pH 7), 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 unit/ml oxidized glutathione reductase and 1mM GSH was prepared. The homogenate was centrifuged at 105,000 for 15minutes at 4 ºC. 0.1 ml of the supernatant was added to 0.8 ml of the reaction mixture and the solution was incubated for 5minutes at 25 ºC. 0.1 ml of 0.25 mM hydrogen peroxide solution was added to initiate the reaction. Absorbance was measured at 340 nm for 5minutes, and an extinction coefficient of 6.22×10^{-3} was used for calculation. The results were expressed as mmol/minute/g tissue. The changes in the absorbance at 340 nm were recorded at 1-minute interval for 5 minutes.

Activity of liver tissue MPO was determined according to Hillegas *et al.* [16]. Tissue samples were homogenized in 50 mM potassium phosphate buffer (PB; pH 6.0), and centrifuged at 42000 ×g (10 minutes); pellets were suspended in 50 mM PB containing 0.5% hexadecyltrimethylammonium bromide. After three freeze and thaw cycles, with sonication between cycles, the samples were centrifuged at 42000 ×g for 10minutes. Aliquots (0.3 ml) were added to 2.3 ml reaction mixture containing 50 mM PB, O-dianisidine and 20 mM H_2O_2 solution. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance measured at 460 nm for 3 minutes. MPO activity is expressed as U/g tissue.

LDH was determined according to Bergmeyer and Bernt [17] and TNF-α was assayed by Enzyme Linked Immunosorbent Assay (ELISA) using the Biosource International California kits according to Senaldi *et al.* [18], as indicators of tissues damage in serum samples.

Cytogenetic analysis

Peripheral-blood micronucleus (PMN) assay

Peripheral-blood smears were prepared according to Holden *et al.* [19]. Drops of blood were collected from tail tip of mice; smears were prepared on cleaned slides, allowed to dry at 37ºC and fixed in absolute methanol for 3 minutes. After fixation, slides were stained with acridine orange and washed twice with phosphate buffer as described by Hayashi *et al.* [20].

Bone-marrow micronucleus (BMN) assay

Bone-marrow slides were prepared as described early [21]. Femur bones were isolated and bone marrow was homogenized with fetal bovine serum (FBS). After centrifugation, the supernatant was discarded and the pellet was re-suspended with the residual FBS. From this suspension, smears were prepared on clean slides and fixed in absolute methanol for 3minutes [22]. The slides were stained with acridine orange as described in PMN assay. The criteria utilized for the BMN analysis were described by Titenko-Holland *et al.* [23].

Frequency of chromosomal aberrations (CAs)

The method was described by Ford and Hamerton [24]. Two hours before sacrifice, each mouse received a single inter peritoneum injection of 0.2ml of 1% colchicine. After the animals were sacrificed by cervical dislocation, one femur was dissected and the bone marrow cells were flushed out with 2ml of 0.9% NaCl solution and then centrifuged. The bone marrow

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suspension was washed with 0.075M KCl cold hypotonic solution and then fixed with 1:3 (acetic acid: methanol). Chromosome preparations were air-dried and stained with 5% Giemsa in phosphate buffer, pH 6.8. Fifty metaphases were scored for each animal and types of aberrations were classified in accordance with Albertini *et al.* [25].

Statistical analysis

Results are given as Mean ± S.E. The statistical analysis of the data obtained was made with the multiple comparisons and analysis of variance (ANOVA). $P<0.05$ was considered to be statistically significant.

RESULTS

Biochemical observations

Gamma-irradiation (4 Gy) induced a significant increase in the level of MDA (↑71 %) and significant decrease in GSH content (↓44 %) compared to control mouse group. Administration of CEO to pre- and post-treated mouse groups exposed to γ-rays resulted in significant ameliorations in MDA levels (↓25&↓19 %, respectively) and significant increases in GSH contents (↑61&↑52 %, respectively) compared with irradiated groups (table 1).

Table 2 showed that, γ-irradiation induced significant decreases in the activities of SOD and GPx (↓25 % & ↓46 %) and a significant increase in the activity of MPO (↑481 %) compared to control group. Pre-and post-treatment of irradiated mice with CEO resulted in significant increases (↑26 % & ↑21 %) and (↑67 % & ↑57 %) in SOD and GPx activities, respectively and a significant decrease (↓34 % & ↓35 %) compared to corresponding irradiated groups.

LDH activity and TNF-α level were significantly increased (↑45 % & ↑200 %) in an irradiated mouse group when compared with the control group, while this irradiation-induced rise in serum LDH activity and TNF-α level, these levels were decreased (↓18 %) & (↓61 %) with pre-treatment with CEO and (↓17%) & (↓52 %) with post- treatment (table 3).

Cytogenetic findings

Administration of CEO to CEO-treated young mouse groups resulted in non-significant changes in all clastogenic and biochemical parameters. Significant increase in PMN (↑269 %) was observed in the irradiated group as compared to control animals. Pre- and post-treated groups showed significant decreases in PMN as compared to irradiated group (↓52 %) & (↓38 %) (table 4).

Table 4 shows the inhibitory effect of chamomile oil on the frequency of BMN produced by γ-rays in bone marrow cells. Clearly, high frequencies were detected in the irradiated group (↑676 %); however, the administration of CEO either pre- or post-exposure to γ-rays gave rise to a statistically significant reduction in the genotoxic damage (↓48 %) and (↓37 %), respectively. Appreciable difference in the BMN frequencies was observed in pre- and post-treated groups.

The results showed a significant increase in number of aberrant cells in the irradiated group (↑35%) but it's reduced to (↓27%) and (↓22%) in post- and pre-treated groups, respectively (figure 1). illustrated that CEO in both pre- and post-treatments significantly decreased the frequencies of all types of CAs which scored in the irradiated group (gaps, chromosome and chromatid breaks, chromatid exchange, and dicentrics).

Table 1. Influence of CEO on LP and GSH contents in liver of γ-irradiated young mice.

Young mouse group	Control (Corn oil)	CEO-treated (1g/Kg body wt)	Irradiation (Corn oil+4Gy)	Pre-treated (CEO+IRR)	Post-treated (IRR+CEO)
MDA (nmol/g tissue)	2.34±21.4	2.13±22.9	4.11±36.6[a]	2.45±27.6[b]	3.11±29.5[b]
GSH (µmol/g tissue)	0.046±0.41	0.041±0.40	0.031±0.23[a]	0.073±0.37[b]	0.059±0.35[b]

IRR= irradiated. [a]P< 0.05 as compared to control. [b]P< 0.05 as compared to IRR.

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Table 2. Influence of CEO on activities of SOD, GPx and MPO in liver of γ-irradiated young mice.

Young mouse group	Control (Corn oil)	CEO-treated (1g/Kg body wt)	Irradiation (Corn oil+4Gy)	Pre-treated (CEO+IRR)	Post-treated (IRR+CEO)
SOD (µg/g tissue)	3.97±43.6	3.73±41.9	4.21±32.5[a]	4.12±40.8[b]	3.62±39.4[b]
GPx (mmol/g tissue)	0.011±0.39	0.021±0.37	0.026±0.21[a]	0.025±0.35[b]	0.031±0.33[b]
MPO (U/g tissue)	1.58±4.7	1.73±14.1	3.14±27.3[a]	1.17±18.1[b]	1.32±17.8[b]

IRR= irradiated. [a]P< 0.05 as compared to control. [b]P< 0.05 as compared to IRR.

Table 3. Influence of CEO on LDH activity and TNF-α content in serum of γ-irradiated young mice.

Young mouse group	Control (Corn oil)	CEO-treated (1g/Kg body wt)	Irradiation (Corn oil+4Gy)	Pre-treated (CEO+IRR)	Post-treated (IRR+CEO)
LDH (U/L)	34.4±606	29.8±589	46.5±877[a]	28.7±711[b]	31.2±731[b]
TNF-α (pg/ml)	0.046±0.41	0.041±0.40	0.031±1.23[a]	0.073±0.37[b]	0.059±0.35[b]

IRR= irradiated. [a]P< 0.05 as compared to control. [b]P< 0.05 as compared to IRR.

Table 4. Effect of CEO on PMN induced by γ-rays in young mouse peripheral-blood and bone marrow cells in the different animal groups.

Young mouse group	Control (Corn oil)	CEO-treated (1g/Kg body wt)	Irradiation (Corn oil+4Gy)	Pre-treated (CEO+IRR)	Post-treated (IRR+CEO)
PMN/1000 erythrocytes cell/mouse	0.25±5.8	0.21±6.1	1.13±21.4[a]	1.32±10.3[b]	1.52±13.2[b]
BMN/1000cell/mouse	0.031±0.66	0.039±0.69	0.365±5.12[a]	0.132±2.65[b]	0.154±3.24[b]

IRR= irradiated. [a]P< 0.05 as compared to control. [b]P< 0.05 as compared to IRR.

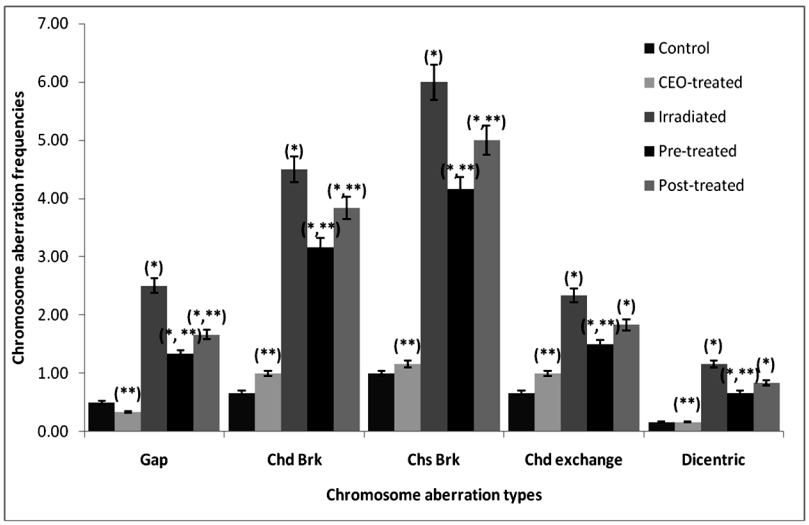

Figure 1. Effect of CEO on chromosome aberration frequencies induced by γ-rays in young mouse bone marrow cells. Chd: Chromatid, Chs: Chromosome, Brk: Break, *P< 0.05 as compared to control, **P< 0.05 as compared to IRR.

DISCUSSION

Chamomile has been used for centuries as a medicinal plant to manage different inflammatory diseases of the gastrointestinal tract and certain mucosal surfaces, such as the oral cavity and anogenital region [26]. Other studies have demonstrated its antioxidant property [27].

Unfortunately, it is not easy to eliminate the source of genotoxicity completely in modern society, the identification and application of well -known anti-mutagens is a valid complementary

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strategy for improving human health [5]. Gamma radiation induces oxidative injury in all animal organs and biomolecules [28] and it can induce clastogenic or cytotoxic effects [29].

Several pathways of radioprotection have been suggested for the mechanism of protective action in mammalian cells against the harmful effects of radiation. These mechanisms include free radical scavenging that protects against ROS generated by radiation and/or hydrogen atom donation to facilitate direct chemical repair at sites of DNA-damage [30]. If ROS remain without being scavenged in the biological system, they may induce biochemical alterations such as inflammation, lipid and protein oxidation, DNA-damage, and certain enzyme activation or inactivation. These observations are consistent with the present study.

MDA is generated by oxidative attack on circulating lipid and acts as a sensitive biomarker of oxidative stress involved in the pathogenesis of various diseases [31] and its formation is increased by γ-irradiation [32]. MDA interacts with DNA, leading to DNA breaks, and decomposition of cellular proteins that finally resulting in cell death [33].

Reduced GSH is the main intracellular antioxidant and has multiple biological functions. It acts directly as ROS scavenger and as a substrate for GPx to reduce H_2O_2 [34]. Reduced GSH is a vital determinant of cellular radio sensitivity because it can react with a variety of electrophilic compounds [35].

SOD, as the first-line defense against the toxic effects of ROS, serves to convert $\cdot O^{-}_{2}$ into H_2O_2 and maintains the oxidant and antioxidant balance [36]. The depletion of GSH contents in irradiated group may be due to the reaction of reduced GSH with free radicals resulting in the formation of thiol radicals that associate to produce oxidized glutathione (GSSG). Moreover, the availability of reduced GSH can also be limited either by a deficiency in the synthesis, enhanced efflux, or inefficient reduction of GSSG [37]. In the irradiated mice, the normal GSH synthesis/repair was disrupted due to the damage of DNA and membranes.

Significant increase in liver, reduced GSH contents and SOD activity and decrease in the LP level were observed in CEO pre- and post-treated animals. GSH offers protection against oxygen-derived free radical following exposure to radiation [38]. The radio protective effects of CEO observed may be attributed to its antioxidant properties. The antioxidant mechanisms of radioprotection and free radical scavenging have been attributed to flavonoids [39]. In addition, phenolic compounds acting as antioxidants may function as terminators of free radical chains and as chelators of redox-active ions that are capable of catalyzing lipid peroxidation [40], so the presence of apigenin as one of the constituents of CEO may contribute to the reduction of MDA. Also, chamazulene may have influenced the CEO effects as it is one of the major components of the oil with a reducing potential competency [41].

Liver SOD activity was remarkably increased when mice were treated with CEO, suggesting that CEO induces SOD activity, which is consistent with the result of Sebai *et al.* [42], they showed that chamomile elevated SOD activity in the liver of rats given chamomile decoction extract against alcohol-induced oxidative stress. To recognize the mechanism of CEO in protecting against the liver damage induced by γ -rays, parallel correlations were found between MDA and GSH contents or SOD activity, implying that liver damage is closely associated with LP induced by γ-rays. Hence, it could hypothesize that the CEO-mediated radioprotection can mainly be attributed to the elimination of oxidative stress by means of direct scavenging ROS and indirectly synthesizing GSH. Also, the radio protective/ anti-oxidative effects of various natural products have also been reported [43].

MPO is an index of tissue neutrophil accumulation [44]. Irradiation-induced increases in MPO activity in the early period of irradiation in a number of exposed animal tissues [45]. In our observations, elevated MPO levels in the liver tissues indicate that neutrophil accumulation contributes to the irradiation-induced oxidative injury and CEO appears to have a preventive effect through the inhibition of neutrophil infiltration and subsequent release of pro inflammatory mediators.

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LDH activity and TNF-α level, as indicators of generalized tissue damage were assayed in the serum samples. LP can disrupt cell compartment, which may contribute to impaired cellular function and necrosis [46] and tissue macrophages responds to any toxic-event by secreting cytokines such as TNF-α [47]. As evidenced in the present study, γ-irradiation resulted in increased serum TNF-α, indicating the role of this cytokine in irradiation-induced toxicity, while depression of the TNF-α response by CEO implicates the inhibitory effect of CEO on the release of this pro-inflammatory cytokine.

In addition, the efficacy of CEO was evident in the case of damage produced by a direct and an indirect mutagen. These characteristics of the extract, as well as its lack of toxicity, suggest that it is applicable to extend the research in animal models, using CEO and the whole tea in order to verify the reported anti-genotoxicity and determine the mechanism of action involved [48]. Other specific constituents of CEO have hardly been studied with respect to protective or pharmacological effects [49]. Among such extracts, the mutagenic and carcinogenic effects on the healthcare persons handling these herbs need to be considered carefully [50].

Mouse is generally preferred over other animals for the evaluation of genotoxicity employing PMN assay as a reliable method for the assessment of clastogenic potential. Micronucleus (MN) arises from acentric chromosome fragments or lagging intact chromosome which fails to fit in the daughter nuclei during the course of nuclear division [51]. These MN cells persist for a longer duration in the peripheral circulation of young mouse [52]. In addition, the main action of mutagenic agents on DNA occurs through the induction of free radicals, which in turn may produce several types of genotoxic damage. Among these, an increase in the rate of MN is recognized [53]. Moreover, γ-rays increased the frequency of MN dramatically and excreted cytotoxic effect of cell proliferation [28].

Apigenin reduced the genotoxic damage, owing to the possible prevention of metabolic activation of γ-rays or scavenging the electrophiles nucleophiles or enhance the DNA repair system or DNA synthesis [54]. In the present study, CEO that contains apigenin significantly reduced PMN in both pre- and post-irradiated groups.

BMN is an indicator of DNA-damage. It considered clastogenic endpoint and any reduction in its frequency gives an indication of the antigenotoxicity of a particular compound [55]. Elevation of the MDA content in the irradiated animals which forms adduct with cellular DNA [56], lead to DNA-damage and subsequently increase the PMN that evident in the present work. *M. chamomilla* is therapeutically used for abolishing oxidative stress. Since constituents of the CEO tested in the present study, like bisabolol and chamazulene, are antioxidants [3], a possible explanation for the inhibitory effect of CEO on the BMN produced by γ-rays is that such compounds trap the free radicals formed during the mutagen biotransformation.

DNA damage is one of the major consequences of radiation exposure onto the biological systems [57] The significant increase in number of CAs that observed in irradiated group and significantly reduced in pre- and post-treated groups contributed to CEO action. With respect to the protective action of CEO on γ -irradiated animals, antioxidants in CEO may provide strong nucleophilic centers, which would enable them to react with electrophilic carcinogens such as ROS; this process would intercept the mutagen and consequently reduce the oxidation on DNA [58]. In addition, chamomile tea was associated with an improved antioxidant status *in vitro* that may contribute reduced mutagenicity [59].

Chamomile, a naturally occurring flavonoid is acting as anti-oxidant has anti-genotoxic action [4]. Previous report on the anti-mutagenic actions of chamomile extract showed that they were due to a combination of two distinctive mechanisms: inhibition of the cytochrome P450-mediated mutagenic metabolite form and interaction with metabolites in such a way to reduce its mutagenic potential [60] and post-irradiation repair mechanisms depend upon the status of endogenous antioxidant enzymes during and after radiation period [61].

The results suggest that the antioxidant and

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free radical-scavenging activities of CEO constitute a likely mechanism for its radio protective effect. The diverse radio protective and antioxidant activities of CEO may be assigned to different chemical constituents.

CONCLUSION

In conclusion, we have demonstrated that CEO induces radio protective and anti-mutagenic Competency on the γ-rays exposed mice either pre- or post-irradiation. The scavenging of γ-rays induced free radicals and the elevation of cellular antioxidants by CEO in irradiated animals could be one of the main leading mechanisms of radiation protection. Up-regulation of enzymes like SOD might be another mechanism of radioprotection by COE. Reduction of LP and elevation of GSH might also contribute to some extent for its radio protective ability.

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***Conflicts of interest*:** *Declared none.*

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