



## Protective effect of kombucha mushroom (KM) tea on chromosomal aberrations induced by gamma radiation in human peripheral lymphocytes *in-vitro*

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**Abstract:** The aim of this study was to investigate the potential radioprotective effect of kombucha mushroom tea (KM-tea) on gamma radiation ( $\gamma$ )-induced chromosomal aberrations (CAs) in human peripheral blood lymphocytes *in vitro*. For this purpose, we used *in vitro* dose-effect relationship, and correlated these data with statistical parameters. CAs were classified into six major types as break, dicentric, acentric, fragment, gap and ring. Mitotic index (MI) and the numbers of aberrant metaphases (AMN) were also calculated for each donor. Six groups of the lymphocytes were prepared by *in vitro* culture according to the standard protocol. Group I (control) did not receive any  $\gamma$ -radiation or KM-tea, Group II (positive control) was treated with 1000  $\mu$ l dose of KM-tea alone, Group III was treated with 5 Gy dose of  $\gamma$ -radiation alone, Group IV was treated with 250  $\mu$ l dose of KM-tea before irradiation, Group V was treated with 500  $\mu$ l KM-tea before irradiation, Group VI was treated with 1000  $\mu$ l KM-tea before irradiation. The results indicated that all KM-tea supplemented lymphocytes had lower frequency of CAs than in the group treated with  $\gamma$ -radiation alone ( $p < 0.05$ ). It was seen that KM-tea had a protective effect against CAs particularly at 500 and 1000  $\mu$ l doses. Besides, MI values increased and AMN decreased after application of KM-tea in a dose/dependent manner. *In vitro* results showed that KM-tea supplementation may decrease the frequency of CAs and its radioprotective action against ionizing radiation is dose-dependent.

**Key words:** Gamma radiation, *In-vitro* chromosome aberration, Kombucha mushroom tea, Radioprotective effect

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### Introduction

Kombucha mushroom tea is a fermented tea beverage produced by fermenting sugared black tea with a kombucha culture known as a mushroom (Dashi *et al.*, 2001). The taste of KM-tea is slightly sweet and acidic (Jayabalan *et al.*, 2008). This tea is called by several different names including Fungus japonicas, Fungo-Japan, Manchurian mushroom tea, Combuch fungus tea, Pichia fermentans, Cembuya orientalis, Tschambucco, Volga spring, Mo-gu, Champinon de longue vie, Teekwass, Kwassan, Kargasok tea and the champagne of life (FDA, 1996; Mayser *et al.*, 1995).

When the fungus is fermented in a mixture containing water, black tea, sugar and vinegar, the microorganisms combine into a complex fermenting culture. This culture produces several compounds that have been considered health tonics over the centuries. KM culture may include microorganism species such as *Saccharomyces ludwigii*, *Schizosaccharomyces pombe*, *Brettanomyces bruxellensis*, *Bacterium xylinum*, *Bacterium gluconicum*, *Bacterium xylinoides*, *Bacterium katogenum*, *Pichia fermentans*, *Candida stellata*, *Acetobacter xylinum*, *Acetobacter aceti*, *Gluconobacter oxydans* (Mrdanovic *et al.*, 2007). It also contains liver detoxifiers, antioxidants, polyphenols, probiotics and free-form amino acids (Stamets, 2008).

This tea is said to have been used for centuries to cure a wide variety of illnesses. Hence, it is used as an alternative therapy (Blanc, 1996). Beneficial effects attributed to consumption of KM-tea have included prevention of a few cancer, relief of arthritis, treatment of insomnia, hemorrhoids, digestive disorders, heart disease, allergies, asthma, decrease of blood pressure, increase of vitality, increase of T cell count and stimulation of regrowth of hair. Because, the tea is believed to stimulate the immune system. Hence, it has become popular among elderly persons (O'Neill, 1994; Timmons, 1994; Steinkraus, 1996; Center of Disease Control and Prevention, 1996; Srinivasan *et al.*, 1997; Sreeramulu *et al.*, 2000).

However, some analyze results demonstrated that KM-tea can become contaminated with potentially harmful microorganisms, such as mould (Mayser *et al.*, 1995; Stamets, 2008). Contamination may potentially produce serious adverse effects. In fact, there have been several reports, indicated that caused problems as nausea, jaundice, shortness of breath, throat tightness vomiting, akathasia, headache, xerostomia, dizziness, liver inflammation, chronic liver disease and neck pain of KM-tea consumption (Peron *et al.*, 1995; Jayabalan *et al.*, 2007).

Consequently, although there are some scientific studies to support the possible benefits of KM-tea, unfortunately, reliable scientific and clinical studies of KM sufficiently have not been published in the literature. In view of these facts, the present study was

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undertaken to find out the protector role of *KM*-tea on CAs  $\gamma$ -radiation-induced in human lymphocytes *in-vitro*.

### Materials and Methods

**Products and chemicals:** RPMI-1640, medium with L-glutamine (cat.01-100-1), fetal bovine serum (heat inactivated, cat.04-121-1), colcemid solution (concentration: 10  $\mu\text{g ml}^{-1}$ , cat.12-004-1), phytohemagglutinin (cat.01-198-1) and penicillin/streptomycin solution (cat.03-031-1) were purchased from Zeydanli Ltd., Ankara, Turkey.

**Preparation of *KM*-tea culture:** *KM*-tea starter culture was obtained from Department of Mushroom Cultivation of Kirikkale University, Turkey and was maintained in sugared black tea. 2% of black tea were added to boiling water and allowed to infuse for about 5 min after which the infusions were filtered through sterile sieve. Sucrose (20%) was dissolved in hot tea and the preparation was left to cool. 200 ml of tea was poured into 1000 ml glass jars that had been previously sterilized at 121°C for 20 min. The cool tea was inoculated with 3% (w/v) of freshly grown tea fungus that had been cultured in the same medium for 7 days and 10% (v/v) of previously fermented liquid tea broth aseptically. The jar was carefully covered with a clean cloth and locked properly. The fermentation was carried out in a dark incubator at 28±3°C for 8-10 days. Later, the culture was centrifuged at 5000 rpm for 20 min aseptically and stored in polypropylene bottles at -25°C until further use (Sajadi, 1998). Besides, we found no evidence of contamination in kombucha products fermented under sterile conditions.

**Experimental protocol:** The present study was carried out on blood samples obtained from 10 health donors. All donors were randomly selected from among healthy non-smoker persons. Of the 10 donors, 5 were male and 5 were female, with a mean age of 23.9±1.7 years (range 20 to 25 years) for the men and 22.4±1.2 years (range 22 to 25 years) for the female. In this study, the methods and techniques applied to donors were carried out favorably to the guidelines set by the World Health Organization (Geneva, Switzerland). All donors signed an informed consent form before participating in the study. The cytogenetical analysis was performed on human peripheral lymphocytes exposed to 5 Gy  $\gamma$ -radiation. Lymphocyte cultures were prepared from peripheral blood samples obtained from 10 healthy donors. Group I (control) did not receive any  $\gamma$ -radiation or *KM*-tea, Group II (positive control) was treated with 1000  $\mu\text{l}$  dose of *KM*-tea alone, Group III was treated with 5 Gy dose of  $\gamma$ -radiation alone, Group IV was treated with 250  $\mu\text{l}$  dose of *KM*-tea before irradiation, Group V was treated with 500  $\mu\text{l}$  dose of *KM*-tea before irradiation, Group VI was treated with 1000  $\mu\text{l}$  dose of *KM*-tea before irradiation. *KM*-tea was added to whole-blood samples at 250, 500 and 1000  $\mu\text{l}$  doses and the samples were kept at room temperature (22°C) for 1 hr before irradiation. Whole-blood samples were irradiated (ATC Cobalt 60 instrument, focus center distance 60 cm) with  $\gamma$ -radiation at a dose 5 Gy for 15 min. in air at room temperature.  $\gamma$ -radiation was applied at a single dose rate after *KM*-tea administration into the culture as described in the literature. The dose of  $\gamma$ -radiation was selected as 5 Gy. This

radiation dose was chosen because it is known to induce a high frequency of CAs in human lymphocytes (Jagetia, 1993). For the analysis of CAs, peripheral blood samples were collected from 10 healthy donors. Approximately 10 ml of venous blood was obtained from each donor, evacuated into heparinized tubes and transported to the laboratory on the same day of collection. After  $\gamma$ -radiation treatment, blood culture was prepared according to Monobe et al. (2003). Briefly, 1.0 ml (approximately  $1.0 \times 10^6$  cells  $\text{ml}^{-1}$ ) of irradiated whole-blood was cultured in 9 ml of a RPMI 1640 medium supplemented with 20% (v/v) heat-inactivated fetal bovine serum, 300 U  $\text{ml}^{-1}$  penicillin-streptomycin, 90  $\mu\text{gml}^{-1}$  PHA and 0.05  $\mu\text{gml}^{-1}$  colcemid. Colcemid was added to the medium at the beginning of the culture in order to obtain enough number of cells at the metaphase in the first cell division. After 53 hr incubation at 37°C, the cultured cells were treated with a hypotonic (100 mM) KCl solution for 15 minute at 37°C and fixed in methanol-acetic acid (3:1). Air-dried slides were made under warm and humid conditions. The slides were stained for 30 min in a coplin jar containing giemsa stain (5%) and observed under a light microscope at a magnification of 500X.

**Mitotic index:** MI was determined as the percentage of dividing cells among 1000 nucleated cells in each culture.

**Aberrant metaphase:** AMN was counted as the number of damaged metaphase cells among 100 metaphases in each culture.

**Chromosomal aberration:** From control, positive control and treatment groups, 100 metaphases were counted for CAs such as break, fragment, dicentric, acentric, gap and ring and considered to be equal. CAs was scored under a binocular light microscope (Japan, Olympus BX51) according to Savage's classification (Savage, 1976).

**Statistical analysis:** The statistical analysis was carried out using SPSS for Windows version 10.0 statistical software (SPSS Inc, Chicago, USA). Statistically significant differences between the groups were compared using one-way analysis of variance (ANOVA) and Duncan's test. The data are displayed as means  $\pm$  standard deviation (SD) and p-values less than 0.05 are considered "statistically significant".

### Results and Discussion

Table 1 presents the results of CAs analysis in mitotic chromosomes of human peripheral lymphocyte cells treated with *KM*-tea alone,  $\gamma$ -radiation alone,  $\gamma$ -radiation + *KM*-tea. The lymphocytes (positive control) treated with 1000  $\mu\text{l}$  doses of *KM*-tea did not show any significant difference in the total number of CAs and AMN compared with the control group ( $p < 0.05$ , Table 2). According to Savage's classification, six structural CAs were determined in the control and the experimental groups. We found a high frequency of CAs such as breaks, fragments, dicentrics, acentrics, gaps and rings (Fig. 1). Fragment type aberrations occurred far more frequently than the other type of aberrations.  $\gamma$ -radiation treatment caused to an increase in the induction of CAs and AM. As expected, lymphocytes treated with 5 Gy dose of

**Table - 1:** The distribution of the different types of CAs observed in human lymphocyte cells (1000 cells for treatment) pretreated with KM-tea (250, 500 and 1000 µl) alone or combination with 5 Gy  $\gamma$ -radiation

Treatment groups	Fragment	Break	Acentric	Dicentric	Gap	Ring
Group I	3.40±0.84 <sup>e</sup>	2.40±0.84 <sup>e</sup>	2.40±0.84 <sup>e</sup>	00.00±0.00 <sup>e</sup>	00.00±0.00 <sup>e</sup>	00.00±0.00 <sup>e</sup>
Group II	3.00±0.67 <sup>e</sup>	2.00±0.67 <sup>e</sup>	2.10±0.74 <sup>e</sup>	00.00±0.00 <sup>e</sup>	00.00±0.00 <sup>e</sup>	00.00±0.00 <sup>e</sup>
Group III	63.50±1.90 <sup>a</sup>	43.50±1.90 <sup>a</sup>	31.80±1.69 <sup>a</sup>	24.80±1.48 <sup>a</sup>	18.50±1.90 <sup>a</sup>	16.10±2.38 <sup>a</sup>
Group IV	53.00±1.63 <sup>b</sup>	35.80±2.20 <sup>b</sup>	24.00±1.56 <sup>b</sup>	19.40±1.35 <sup>b</sup>	14.80±1.40 <sup>b</sup>	11.70±1.77 <sup>b</sup>
Group V	44.10±1.79 <sup>c</sup>	27.70±2.50 <sup>c</sup>	21.60±1.96 <sup>c</sup>	14.80±1.62 <sup>c</sup>	11.40±1.35 <sup>c</sup>	9.30±1.42 <sup>c</sup>
Group VI	32.40±2.46 <sup>d</sup>	22.70±2.36 <sup>d</sup>	15.00±1.49 <sup>d</sup>	11.60±1.65 <sup>d</sup>	7.30±1.42 <sup>d</sup>	6.30±1.42 <sup>d</sup>

Values presented as mean±SD (n=10). Means denoted with different superscripts are within the same column are statistically significant (p<0.05)

**Table - 2:** The percentage of MI (10000 cells for treatment) and AMN (1000 cells for treatment) observed in human lymphocyte cells pretreated with KM-tea (250, 500 and 1000 µl) alone or combination with 5 Gy  $\gamma$ -radiation

Treatment groups	AMN	(%)	MI	(%)
Group I	01.60±0.70 <sup>e</sup>	(0.16)	811.30±6.41 <sup>a</sup>	(8.113)
Group II	01.30±0.67 <sup>e</sup>	(0.13)	839.30±7.96 <sup>b</sup>	(8.393)
Group III	42.90±1.79 <sup>a</sup>	(4.29)	412.90±7.43 <sup>f</sup>	(4.129)
Group IV	38.40±2.72 <sup>b</sup>	(3.84)	489.30±8.78 <sup>e</sup>	(4.893)
Group V	29.80±1.87 <sup>c</sup>	(2.98)	552.80±16.56 <sup>d</sup>	(5.528)
Group VI	20.50±2.27 <sup>d</sup>	(2.05)	603.50±5.25 <sup>c</sup>	(6.035)

All values are the mean±SD (n=10). Means denoted with different superscripts are within the same column are statistically significant (p<0.05), AMN = Damaged metaphase / 100 as counted metaphase, MI = Dividing cell / 1000 as counted cell

$\gamma$ -radiation presented a high frequency in both the total number of CAs and AM compared with the control and the KM-tea-treated groups (p<0.05). Besides, there was a considerable decrease in MI and Duncan's test showed that this decrease was statistically significant (p<0.05).

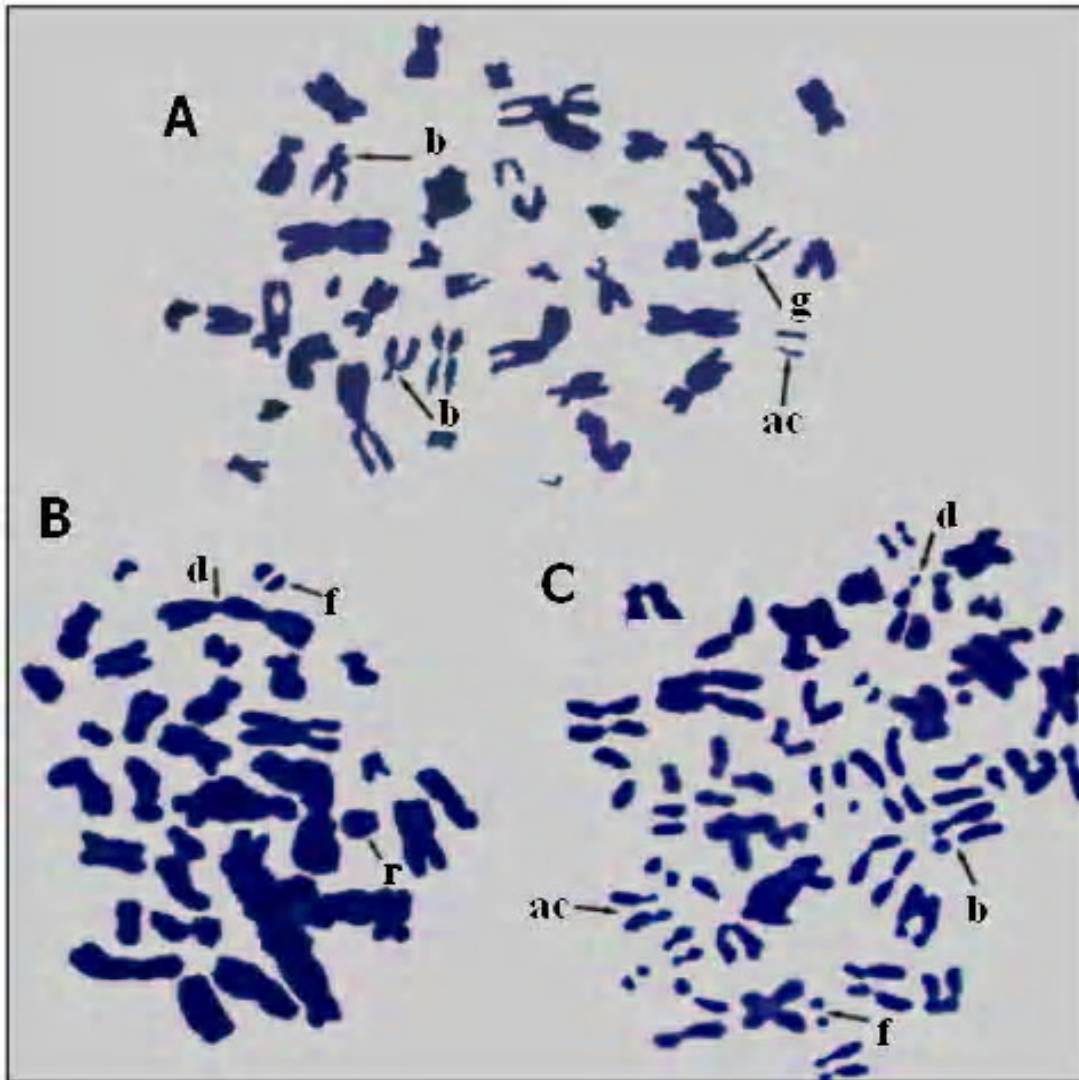
However, pretreatment of cells with KM-tea resulted in a significant reduction in the frequency of CAs and AM before radiation-exposure at the dose of 5 Gy. The number of CAs and AM showed a tendency to decrease in lymphocytes treated with 250, 500 and 1000 µl doses of KM-tea when compared to  $\gamma$ -radiation-only treated group. In 1000 µl dose of KM-tea, this reduction for breaks, fragments, dicentric, acentrics and AM was by at least 2-fold compared with the radiation group. In addition, MI data for KM-tea treatment groups presented a rising trend.

In recent years, genetic monitoring of populations exposed to potential mutagens is an early warning system for genetic disease as cancer (Grover *et al.*, 2003). The formation of CAs and micronuclei is a widely used and accepted as genetic end-point of genotoxicity (Meszaros *et al.*, 2004; Celik and Akbas, 2005; Mozdarani and Kamali, 1998). In the present study, the levels of  $\gamma$ -radiation-induced genotoxicity were determined using as an indicator the CAs test in human lymphocytes. The experimental data obtained in the present study showed that the frequency of CAs induced by  $\gamma$ -radiation in human lymphocytes *in vitro* was significantly affected in the presence or absence of KM-tea. Table 1 shows the mean CAs levels in all 6 groups. The frequency of CAs in the radiation group (Group III) was significantly higher than those in the control and positive controls (p<0.05). The highest MI and

lowest AMN were also observed in these 2 groups compared to the other groups. Besides, no significant differences in the number of CAs and aberrant metaphases (AM) were observed between the control and positive controls (p>0.05). Chromosome-type aberrations such as breaks, rings, dicentrics, acentrics, fragments, gaps and rings were found in lymphocytes treated with  $\gamma$ -radiation. The number of fragments was significantly higher than the other-types of CAs. The incidence of CAs was found fragment > break > acentric > dicentric > gap > ring.

If we consider the similar studies, our results are found to be in close agreement with previously published data. Researchers have shown that  $\gamma$ -radiation is genotoxic and causes genetic mutations, DNA damage, micronuclei formation, sister-chromatid exchanges, increase the frequency of abnormal cells, decrease the density of MI and CAs such as chromosome breaks, dicentrics, centric rings, acentric fragments and translocations *in vivo* and *in vitro* (Watson and Gillies, 1975; Liniecki *et al.*, 1977; Puerto *et al.*, 2001; Krishnaja and Sharma, 2004; Cavusoglu *et al.*, 2009; Cavusoglu and Yalcin, 2009).

The number of CAs and AM in the group exposed to  $\gamma$ -radiation alone were higher than those in the groups supplemented with KM-tea. The MI levels were also lower than those in the supplemented groups, and the differences were statistically significant. Detailed results for MI and AMN are showed in Table 2. In the absence of KM-tea, 5 Gy dose of  $\gamma$ -radiation caused a significantly increase in the numbers of CAs and AM and a decrease in the number of MI. The supplementation with different doses of KM-tea again caused a significantly decrease in the number of CAs and AM



**Fig. 1:** Chromosomal aberrations (CAs) induced by 5 Gy  $\gamma$ -radiation (ac: acentric, b: break, d: dicentric, f: fragment, g: gap, r: ring) magnification X 500

and an increase in the level of MI. There was a strong dose-effect relationship between CAs and *KM*-tea doses. For the groups supplemented with *KM*-tea, the maximum effect of supplementation was seen at 1000  $\mu$ l dose of *KM*-tea.

In conclusion, supplementation with *KM*-tea resulted in beneficial effects against genotoxicity induced by  $\gamma$ -radiation in human lymphocytes *in vitro*. It was shown that *KM*-tea had a protective effect on CAs and this effect was dose-dependent. The protector effect of *KM*-tea on  $\gamma$ -radiation-induced CAs may be explainable with the antioxidant properties of *KM*-tea. Although it is not a general rule, antioxidants and *KM*-tea share similar mechanisms of protection against the toxicity. In the body, antioxidants act as free radical scavengers and they trap the free radicals and give up own electrons. Thus, they protect against free radical oxidation molecules such as protein, lipid, enzyme, chromosome and DNA (Feri, 1994; Halliwell *et al.*, 1995; Siddique *et al.*, 2007; Shadab *et al.*, 2006). An excess availability of free radicals accompanied by a reduction in the

capacity of the natural antioxidant systems leads to cellular dysfunction and death. Superoxide ( $O_2^-$ ), and hydroxyl radical (OH) are the predominant cellular free radicals, while hydrogen peroxide ( $H_2O_2$ ) and peroxynitrite ( $ONOO^-$ ), although not themselves free radicals, aid substantially to the cellular redox state. The cytotoxicity of free radicals is related to the ability of these molecules to oxidize cell constituents, particularly lipids and nucleic acids (Ilhan *et al.*, 2004; Cheun *et al.*, 2007). *KM*-tea is known to be efficient in helping to treat or prevent diseases associated with free radicals. The antioxidant effects of *KM*-tea can be explained by presence of compounds such as tea polyphenols, flavonols, catechins, caffeine, catechin gallates, adenine, theobromine, theophylline, gluconic acid, glucuronic acid, lactic acid, tannins, gallotannin, small amounts of aminophylline and a yellow volatile oil that is solid at ordinary temperatures and has strong aromatic odor and taste (Zi, 1993). It also contains vitamins, aminoacids, antibiotics and micronutrients produced during fermentation. Antioxidant activity

of KM-tea is dependent on the structure of the free-radical scavenging compounds, the substituents present on the rings of flavonoids and the degree of polymerization. It is known that epicatechin and epicatechin polymers are better antioxidants than the catechin and catechin polymers (Saint-Cricq de Gaulejac *et al.*, 1999). Besides, one of the most important metabolites from therapeutic point of view is glucuronic acid, a carrier of detoxification activity of KM (Cvetkovic and Markov, 2002). The major polyphenolic components, glucuronic acid, catechin and epicatechin provide the fundamental structural criteria for being a good antioxidant (Jayabalan *et al.*, 2008). It is said that these compounds block the action of activated oxygen molecules, known as free radicals, that can damage cells (Sajadi, 1998). The probable mechanisms by which these compounds act might include inhibition of promutagen activation, the inactivation or detoxification of reactive forms of mutagens and carcinogens, induction of DNA repair, and inhibition of promotion, invasion and metastasis of tumor cells (Halder *et al.*, 2006). Human laboratory tests and animal studies have shown that KM-tea contains antiradical or antioxidant properties. In a study conducted in Russia by the Central Oncological Research Unit and the Russian academy of Sciences in Moscow found that the daily consumption of KM-tea was correlated with an extremely high resistance to cancer. It is claim that this tea can detoxify the body and enhance the immune system thereby improving the body's defenses, especially in the early stages of cancer (Dufresne and Farnworth, 2000). Jayabalan *et al.* (2007) suggested that KM-tea prevents paracetamol induced hepatotoxicity and chromate induced oxidative stress in albino rats. In a similar study, KM-tea's been shown to protect against oxidative stress and improve liver function in rats (Sai *et al.*, 2000). In a study designed by Vijayaraghavan *et al.* (2000) found that KM-tea had not toxic effects on body weight ratio and histological symptoms in rats fed with KM-tea for 90 days.

In conclusion, the results of the present study clearly confirmed that  $\gamma$  - radiation induces CAs formation in human lymphocyte cells. But, supplementation with KM - tea can protects against  $\gamma$  - radiation toxicity, by reduction effects of free radicals. Therefore, antioxidant role of KM-tea may be used as a "genotoxicity-limiting agent" to reduce environmental effects of radioactive agents in the near future.

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