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Protective effect of kombucha mushroom (KM) tea on phenol-induced cytotoxicity in albino mice

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Abstract: *The present study was carried out to evaluate the protective role of kombucha mushroom (KM) tea on cytotoxicity induced by phenol (PHE) in mice. We used weight gain and micronucleus (MN) frequency as indicators of cytotoxicity, and supported these parameters with pathological findings. The animals were randomly divided into seven groups: (Group I) only tap water (Group II) 1000 µl kg^{-1} b. wt KM–tea, (Group III) 35 mg kg^{-1} body wt. PHE (Group IV) 35 mg kg^{-1} body wt. PHE + 250 µl kg^{-1} b. wt KM–tea (Group V) 35 mg kg^{-1} b. wt PHE + 500 µl kg^{-1} b. wt KM–tea (Group VI) 35 mg kg^{-1} b. wt PHE + 750 µl kg^{-1} b. wt KM–tea, (Group VII) 35 mg kg^{-1} b. wt PHE + 1000 µl kg^{-1} b. wt KM–tea, for 20 consecutive days by oral gavage. The results indicated that all KM–tea supplemented mice showed a lower MN frequency than erythrocytes in only PHE–treated group. There was an observable regression on account of lesions in tissues of mice supplemented with different doses of KM–tea in histopathological observations. In conclusion, the KM–tea supplementation decreases cytotoxicity induced by PHE and its protective role is dose-dependent.*

Key words: *In-vivo micronucleus assay, Kombucha mushroom tea, Phenol toxicity, Pathology, Weight gain*
PDF of full length paper is available online

Introduction

Kambucha mushroom tea is a popular fermented beverage made by black tea, sugar and a kombucha culture known as a mushroom (Dashi *et al*., 2001). It is called by several different names: Fungus japonicas, Fungo–japon, Manchurian mushroom tea, Kombucha 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, 1995; Mayser *et al*., 1995).

When the fungus is fermented in a mixture containing water, black or green 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 several species of yeast and bacteria such as *Saccharomycodes ludwigii, Schizosaccharomyces pombe, Brettanomyces bruxellensis, Bacterium xylinum, Bacterium gluconicum, Bacterium xylinoides, Bacterium katogenum, Pichia fermentans* and *Candida stellata*. It also contains liver detoxifiers, antioxidants, polyphenols, probiotics, and free–form amino acids (Stamets, 1995; Teo *et al*., 2004).

This tea is said to have been used for centuries to cure a wide variety of illnesses. Beneficial effects attributed to consumption of KM–tea have included prevention of a few cancers, 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 (Yang *et al*., 2002). Because the tea is believed to stimulate the immune system, so it has become popular among elderly persons (O' Neill, 1994; Timmons, 1994; Steinkraus, 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, 1995). 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; Srinivasan *et al*., 1997; Sajadi, 1998; Jayabalan *et al*., 2007).

Consequently, although there are some scientific studies to support the possible benefits of this 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 undertaken to find out the protector role of KM–tea in albino mice treated with phenol (PHE).

Materials and Methods

A total of 42 of male mice (*Mus musculus var. albino*) of 12-14 week old were used for MN analysis and change in body weight. Healthy mice with mean body weight 29.03±1.45 g were obtained from the Animal Research Center of Refik Saydam Hifzissiha

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Institute (Ankara, Turkey). The mice were housed in stainless–steel cages under controlled conditions of 22±3°C, 55±5% relative humidity and a 12–hr light/dark cycles throughout the experiment. They were acclimatized 1 week prior to the planned experiments and fed with standard pellet diet (Samsun Food Industry–Samsun, Turkey) and water *ad libitum*. The initial and final weights of all mice were measured by electronic balance. The peripheral blood samples were collected from mice at the end of 20th day and determined the frequency of micronucleus (MN) in erythrocyte cells. Ethical standards of Kirikkale University–Faculty of Veterinary Medicine and guidelines of World Health Organisation (Geneva, Switzerland), were followed.

Preparation of KM-tea culture: 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. Two hundred 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. KM–tea starter culture was obtained from Department of Mushroom Cultivation of Kirikkale University–Turkey and was maintained in sugared black tea.

Experimental protocol: Albino mice were divided into seven groups each consisting of six animals: Group I (control): only tap water, Group II: only 1000 µl kg^{-1} b. wt KM tea, Group III: 35 mg kg^{-1} b. wt PHE, Group IV: 35 mg kg^{-1} b. wt PHE + 250 µl kg^{-1} b. wt KM–tea, Group V: 35 mg kg^{-1} b. wt PHE + 500 µl kg^{-1} b. wt KM–tea, Group VI: 35 mg kg^{-1} b. wt PHE + 750 µl kg^{-1} b. wt KM–tea, Group VII: 35 mg kg^{-1} b. wt PHE + 1000 µl kg^{-1} b. wt KM–tea, once a day for 20 consecutive days by oral gavage. The PHE (PDH-England) solution contained 35 mg kg^{-1} b. wt PHE were prepared daily in distilled water.

MN assay: The Mouse-Erythrocyte-Micronucleus (Mus–EMN) assay was used throughout this study. Mus–EMN assay is a modified mouse–MN test which conventionally scores the MN frequencies in bone marrow polychromatic etythrocytes (PCE). In Mus–EMN assay, the MN frequency is scored in mature normachromatic erythrocytes (NCE) in the circulating blood obtained from the tail of the mouse. The mice were fainted under ether anesthesia and the blood samples were collected from a small puncture on the tail of the mouse. About 5 µl of peripheral blood collected from each mouse was immediately mixed with 3% ethylene diamine tetraacetic acid (EDTA) solution in a cell-well and smeared onto a clean slide. The erythrocytes were fixed in 70% methanol for 2 min and allowed to dry in a high temperature (40°C) incubator overnight. Then, slides stained with 5% May-Grunwald Giemsa (Sigma) staining (Te-Hsiu *et al*., 1995). Usually 3-4 slides were made and repeatedly double scored by two different observers. From each slide, 1000 normachromatic erythrocytes (NCE) were scored for the presence of MN under oil immersion at 100X magnification using a binocular light microscope (Japan, Olympus BX51) and MN cells were photographed Abstract Article–INCLUDEPICTURE "http://www.sciencedirect.com/scidirimg/icon_pdf.gif" * Mergeformatinet - View Record in Scopus Cited By in Scopus (50) at a magnification of X500. The criteria suggested by Schmid (1975) for recognizing MN were followed.

Histopathological examinations: Tissue samples from heart, lung, liver, kidney, stomach and intestine were fixed in 10% neutral buffered formalin solution. After the routine histopathological processing, tissue samples were embedded in paraffin wax and sectioned at 6 micron. All sections were stained with Hematoxylin and Eosin (H&E). Histopathological changes were semiquantitatively assessed under the light microscope. We investigated five slides for each mouse in seven group. Histopathological changes were graded as (–) no changes, (+), (++) and (+++) indicating mild, moderate and severe changes, respectively (Revathi and Yogananda, 2006).

Statistical analysis: For the statistical analysis, differences between the groups were tested by analysis of variance (ANOVA) and Duncan test using SPSS for Windows version 10.0. The data were displayed as means ± standard deviation (SD) and p values less than 0.05 were considered significant.

Results and Discussion

MN frequency: Microscopic examination of erythrocyte cells showed that there were several MN formations in the control and KM–tea treated groups. The MN frequency of erythrocytes belonging to KM–tea treated group was fairly similar to control group. PHE–treatment showed a significant increase in the number of MN formation (Fig. 1). Detailed information related with the MN frequency was showed in Table 1 and Table 3. The highest frequency of MN was observed in mice treated with only PHE and least frequency of MN was observed in mice treated with different dose of KM–tea. The animals treated with different doses of KM–tea showed a significant reduce ($p<0.05$) in the frequency of MN when compared with the animals treated with PHE alone. MN frequency decreased with rising of the KM–tea doses. There was a significant dose–effect relationship between the MN frequency and KM–tea. MN frequency in the mice treated with 750 and 1000 µl/kg b. wt doses of KM–tea was lower than those in mice treated with 250 and 500 µl kg^{-1} b. wt doses of KM–tea. There was statistically significant difference between control and treatment groups for MN frequency ($p<0.05$). Besides, there were statistically significant differences among 250-1000 µl kg^{-1} b. wt doses of KM–tea ($p<0.05$), but differences between 250-500 µl kg^{-1} b wt. doses were not statistically significant.

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Table - 1: Effect of KM–tea on the frequency of MN induced by PHE in mice erythrocyte cells

Treatment time (day)	Groups	Number of scored cell	Minimum	Maximum	Average ± SD
20	Group I	1000	2	3	2.20±0.45
20	Group II	1000	1	3	1.80±1.10
20	Group III	1000	50	55	51.60±2.07
20	Group IV	1000	40	43	41.40±1.14
20	Group V	1000	38	41	39.60±1.34
20	Group VI	1000	28	32	30.00±1.58
20	Group VII	1000	20	25	21.80±1.92

Values presented as mean±SD (n=5)

Table - 2: Effect of KM–tea on mean body weights of mice treated with PHE

Treatment time (day)	Groups	Initial weight (g)	Final weight (g)	Difference (g)
20	Group I	29.00±1.00	40.80±0.83	11.80
20	Group II	29.20±0.44	43.20±0.83	14.00
20	Group III	29.00±0.70	25.00±0.70	4.00
20	Group IV	28.80±0.44	30.00±0.70	1.20
20	Group V	28.60±0.54	31.80±0.83	3.20
20	Group VI	29.60±0.54	34.80±0.44	5.20
20	Group VII	29.00±1.00	36.80±0.83	7.80

Values presented as mean±SD (n=5)

Table - 3: Statistical comparision of data belonging to MN frequency and body weight determined in treatment group of mice

Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
MN frequency	2.20 ± 0.45^{e}	1.80 ± 1.10^{e}	51.60 ± 2.07^{a}	41.40 ± 1.14^{b}	39.60 ± 1.34^{b}	30.00 ± 1.58^{c}	21.80 ± 1.92^{d}
BodyWeight(g)	40.80 ± 0.83^{b}	43.20 ± 0.83^{a}	25.00 ± 0.70^{g}	30.00 ± 0.70^{f}	31.80 ± 0.83^{e}	34.80 ± 0.44^{d}	36.80 ± 0.83^{c}

Values presented as mean±SD (n=5). Means denoted with different superscripts are within the same line are statistically significant

Table - 4: Histopathological evaluation in tissues of mice treated with KM–tea and PHE

Histopathological change	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
Lung -							
Hyperemia and emphysema of the lung	–	–	+ + +	+ + +	+ +	+ +	+
Liver -							
Hepatic hyperemia	–	–	+ + +	+ + +	+ +	+ +	+
Hepatic hemorrhage	–	–	+ + +	+ ++	+ +	+ +	+
Hepatic degeneration	–	–	+ + +	+ + +	+ +	+ +	+
Heart -							
Miyocardial hyperemia	–	–	+ + +	+ + +	+ +	+ +	+ +
Miyocardial hemorhage	–	–	+ + +	+ + +	+ + +	+ +	+ +
Stomach -							
Gastric hyperemia	–	–	+ + +	+ + +	+ +	+ +	+
Gastric hemorrhage	–	–	+ +	+ +	+	+	+
Intestine -							
Intestinal hyperemia	–	–	+ + +	+ ++	+ +	+ +	+
Intestinal hemorrhage	–	–	+ +	+ +	+	+	+
Kidney -							
Renal hyperemia	–	–	+ + +	+ ++	+ +	+	+
Renal hemorrhage	–	–	+ +	+ +	+	+	+
Tubulary degeneration of kidney	–	–	+	+	+	+	+

(–): no change, (+): mild change, (++): moderate change, (+++): severe change

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Fig. 1: The appearance of erythrocyte cells in blood tissue of group III treated mice. Arrow: a micronucleated erythrocyte. Giemsa staining. X500

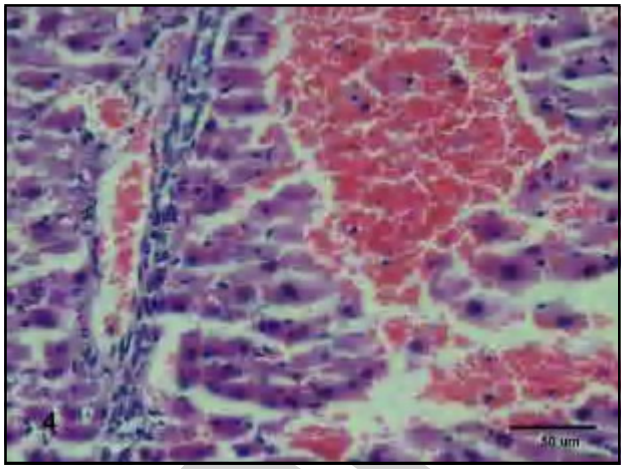

Fig. 4: Section of kidney in group III treated mice. Section shows hyperemic and hemorrhagic foci. H&EX50

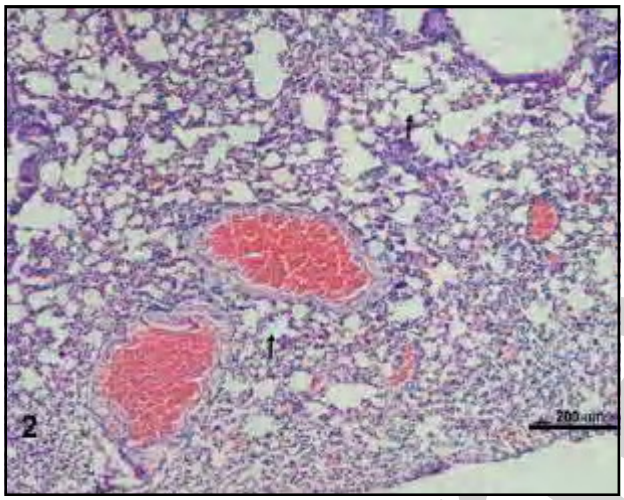

Fig. 2: Section of lungs in group III treated mice. Arrows: hyperemic vessels and alveolar emphysema. H&EX200

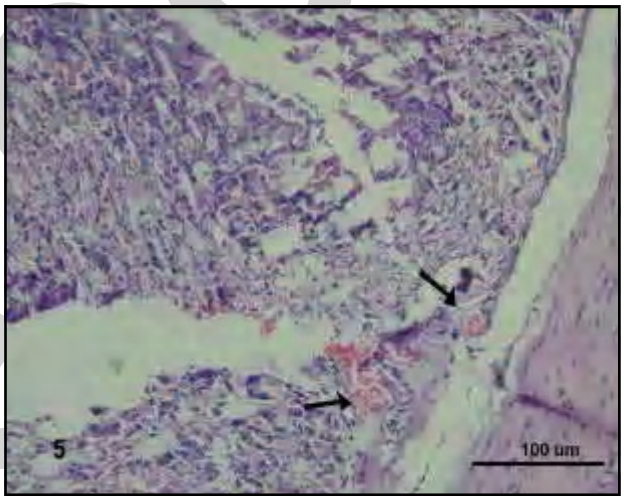

Fig. 5: Section of stomach in group III treated mice. Arrows shows characteristic hyperemic and hemorrhagic vessels. H&EX100

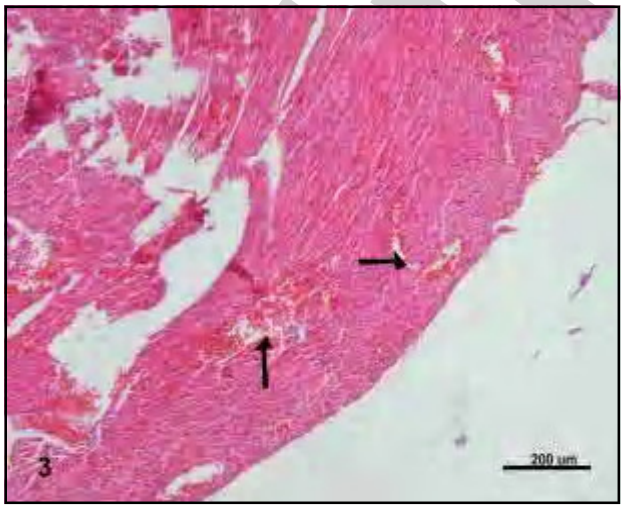

Fig. 3: Section of heart in group III treated mice. Arrows show severe hyperemic and hemorrhagic vessels. H&EX100

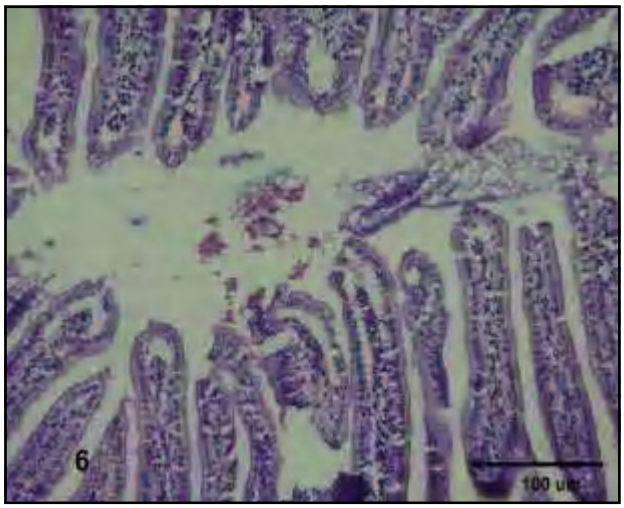

Fig. 6: Section of small intestine in group IV treated with mice. Section shows bleeding on the mucosal surface. H&EX100

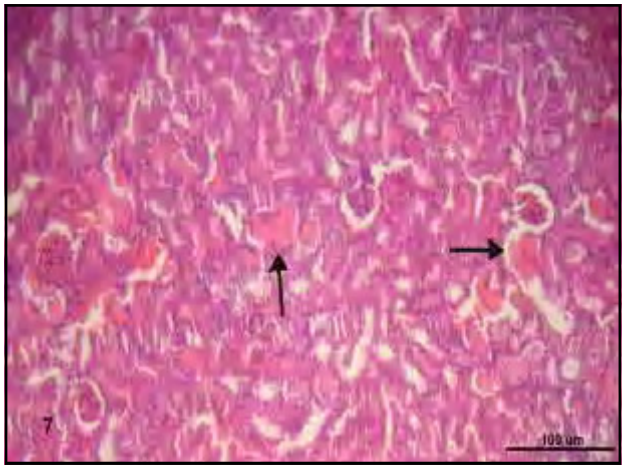

Fig. 7: Section of kidney in group IV treated mice. Arrows show dilated tubulary lumens filled with albuminoid content. H&EX100

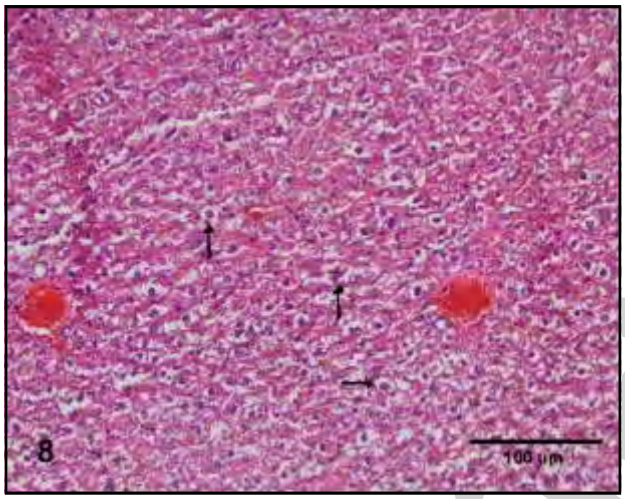

Fig. 8: Section of liver in group VII treated mice. Arrows show regressing in the rate of hemorrhage with hepatocyte degeneration. H&EX100

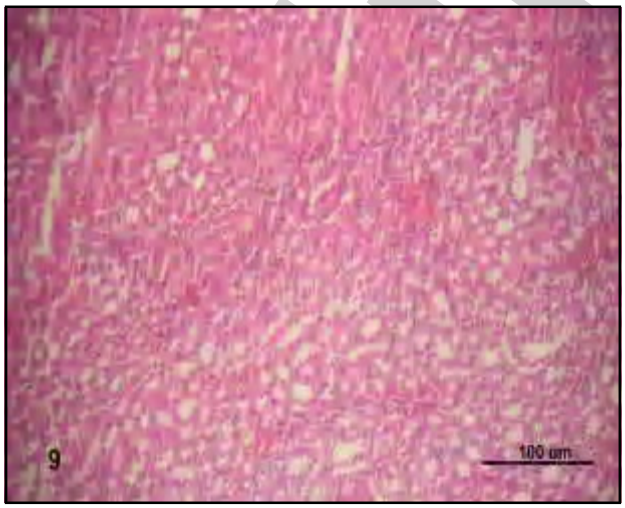

Fig. 9: Section of kidney in group VII treated mice. Shows decreasing in the rate of dilated tubules. H&EX100

Weight gain: To investigate the effects of PHE and KM–tea on the body weight of mice, the initial and final weights of mice by an electronic balance were calculated. The results related with the weight gain were given in Table 2, 3. These data showed that PHE treatment significantly prevented the weight gain of mice. The highest weight gain was observed in group II at the end of experimental period. The least body weight was observed in mice treated with only PHE. In control and group II, the weights of all mice increased about 11.80, 14.00 g according to initial weight, respectively. The weights of mice exposed to only PHE showed decrease of about 4.00 g according to initial weight. But, KM–tea supplement caused again rising in body weight when compared with mice exposed to PHE alone. In mice supplemented with 250, 500, 750 and 1000 µl kg^{-1} b. wt doses of KM–tea, the body weight increased about 1.20, 3.20, 5.20 and 7.80 g according to initial weight, respectively. A parallel correlation was determined between increases in KM–tea doses with increase in weight gain. Besides, these increases were statistically significant ($p<0.05$).

Pathological findings: Detailed information related with the histopathological findings was showed in Table 4.

No histopathological changes were observed in mice of group I and group II. There were severe lesions in tissues of group III and IV mice. Many blood vessels were dilated and hyperemic in lung (Fig. 2). There was emphysema in the alveoli. Hyperemic vessels were also seen in heart tissue. In addition, hemorrhagic foci were detected in myocard (Fig. 3). In liver, central veins and sinusoids were dilated because of intense hyperemia (Fig. 4). There was also hemorrhage in these regions. Many hepatocytes were swollen and pycnotic nuclei were detected in some hepatocytes. Hyperemic vessels and hemorrhagie were also detected in the gastric and intestinal propria (Fig. 5,6). In kidney, there was hyperemia and hemorrhagia in the medulla. Tubulary dilatation was detected and they were filled with albuminoid content (Fig. 7). In group V, histopathological changes were similar to those from group III. But, there was a decrease in the severity of the lesions. Especially, the rate of hemorrhagic lesions decreased or regressed in this group. Nevertheless, there was a limited regression in the rate of swollen hepatocytes. In group VI, histological results were similar to those from group V. But, the regression rate of the histopathological lesions in tissues fairly increased according to group V. In group VII, the regression in the severity of histopathological lesions was fairly prominent in proportion to group V and VI. There was limited hyperemi in the liver (Fig. 8), kidney and myocardial tissues (Fig. 9). It was detected an observable regression in the hemorrhagic lesions, and decreasing in the rate of dilated tubules.

Genetic monitoring of populations exposed to potential mutagens is an early warning system for genetic disease as cancer. The most frequently used genetic endpoints are chromosomal aberrations and MN frequency (Mozdarani and Kamali, 1998; Celik and Akbas, 2005). The result of MN assay indicated that no statistical difference was observed between MN scores in

erythrocytes obtained from the control and KM–tea–treated groups. There were several MN formations in erythrocytes of mice belong to either group. However, results revealed a significant increase in frequency of MN in peripheral blood erythrocytes obtained from mice treated with PHE. These data are in agreement with the previous reports obtained on the potential effects of PHE. For example, Spencer *et al*. (2007) observed a statistically significant increase in MN frequency at the 300 mg kg^{-1} PHE dose. Similarly, using the bone marrow MN test, a significant increase in MN was reported in mice treated with 265 mg kg^{-1} body wt dose of PHE (IPCS, 1994).

The frequency of MN in all groups supplemented with KM–tea was much lower than those treated with PHE alone. In mice treated with KM–tea the MN frequency decreased parallel with the increasing of KM–tea doses. In other words, the frequency of micronucleated erythrocytes decreased with KM–tea supplementation. The results indicate that KM–tea could reduce mutagenic effects of PHE.

In this study we also investigated the changes in body weight of PHE and KM–tea treated mice. As a result, significant differences were observed in weight gains of mice treated with PHE when compared with the control and KM–tea treated groups. The mean body weights of mice in all PHE treatment groups were lower than those of the controls. The highest level of body weight was observed in positive control group mice and least level of body weight was observed in mice treated with PHE alone.

Supplementation with different doses of KM–tea was associated with an increase in weight gain. In KM–tea–supplemented group, the largest effect of supplementation was seen at 1000 µl/kg b. wt dose of KM–tea. 250, 500, 750 and 1000 µl kg^{-1} b. wt doses of KM–tea caused 20, 27, 39 and 47% increase respectively of body weight in comparison group treated with PHE alone. These results may relate to the metabolism rate of the mice. Namely, with exposure to PHE in feeding period, the metabolism and diets of mice may be changed and body weights of mice may be decrease. As a result, the metabolisms of mice enhance for the removal of PHE. So the energy consumption increases and the mice loss weight. This information is parallel with other data available so far. Many epidemiological studies showed that there is a clear relationship between the weight decrease and exposure to PHE. For example, dose–related decreases in body weight and water consumption when compared with the controls were reported in male and female rats exposed to PHE. In a similar study, the decrease in final body weights when compared to controls in rats and mice treated with 10,000 ppm PHE was observed (Anonymous, 2002).

Besides, the cause another of this weight loss may be related with digestion system damage. In a previous study reported that the third of the mucosal surface of the stomach was thickened and necrotic in mice exposed to difference doses of Allyl Isothiocyanate (NTP, 1982). It has been also reported that PHE is cause severe corrosive injury to the mouth, throat, esophagus and stomach, with bleeding, perforation, scarring, or structure formation as potential sequelae (Luttrell, 2003; Rana and Verma, 2005). This situation may be reduced water and food consumption in mice, and mice loss weight. In the present study, prominent histopathological changes such as hyperemia (in lung, myocard, liver, stomach, intestine and kidney tissues), hemorrhage (in myocard, liver, stomach, intestinal tissues), degenerative changes (hepatic and renal tissues) and emphysea (in the lung) were observed in only PHE–treated group. In groups supplemented with KM–tea, limited hyperemia was seen in pulmonary, gastric, myocardial and intestinal blood vessels. Although there was no prominent regression in group IV, it was seen a prominent decreasing in the severity of the hemorrhagic lesions in the stomach, intestine, liver and myocardial tissues of mice supplemented with 500, 750 and 1000 µl kg^{-1} b. wt doses of KM-tea.

All these results suggest that this tea may prove useful in reducing of the toxic effects induced by chemical agents as PHE. The protective effects of KM–tea on PHE–induced cytotoxicity may be attributed to its antioxidant activity. Although it is not a general rule, antioxidants and KM–tea share similar mechanisms of protection against the toxicity (Cavusoglu and Yalcin, 2009; Edwin *et al*., 2002). 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 therapeutical point of view is glucuronic acid, a carrier of detoxification activity of kombucha (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). 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 (Dufresne and Farnworth, 2000). This antioxidant role of KM–tea was reported by several biomonitoring studies. For example, 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

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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 present study demonstrated that PHE-induced toxicity causes pathological injuries in lung, heart, stomach, intestine, liver and kidney tissues of mice. It was also demonstrated that KM–tea may afford the protection against PHE toxicity.

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