

Evaluation of the toxicity and safety of the antioxidant beverage effective microorganisms-X (EM-X) in animal models

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Abstract

The acute and chronic toxicity tests and the mutagenic test of the extracts from the fermentation of plants with effective microorganisms (EM-X) were performed in the mouse and the rat. In acute toxicity test, mice were orally treated three times per day with 20-fold of concentrated EM-X for 7 days. For chronic toxicity test, the rats were orally treated with original EM-X once a day for 90 days at the dosages of 180, 120 or 60 ml/kg. At the levels tested EM-X did not lead to significant changes in food consumption, body weight, behaviors and stools. Hematological assays on red blood, white blood cell, hemoglobin, platelets, lymphocyte, granulocyte, middle cell and coagulation time and the biochemical assays on aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, blood urea nitrogen, total protein, albumin, glucose, total bilirubin, creatinine and total cholesterol did not show abnormal changes. The histological inspection of principal organs of the heart, liver, spleen, lung and kidney did not show significant pathological changes. The delaying toxic reactions were detected 2 weeks after administration of EM-X was stopped. The mutagenic test showed that EM-X did not cause mutagenesis and tests of micronucleus of bone marrow cell and sperm shape abnormality upon EM-X were negative. The maximal tolerance dose of EM-X was calculated to be 1800 ml/kg BW in the mouse and rat. Thus, oral administration of EM-X does not present acute and chronic toxicity and mutagenic effects in the animals.

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1. Introduction

The beverage effective microorganisms-X (EM-X) is a novel antioxidant cocktail derived from fermentation of unpolished rice, papaya and sea weeds with grouped effective microorganisms of lactic acid bacteria, yeast and photosynthetic bacteria. It contains mixed-extracts of

plants and effective microorganisms. EM-X contains over 40 minerals (antioxidants such as flavonoids, kaempferol, panaxin, quercetin, lycopene, oryzanol, ascorbic acid, tocopherol, ubiquinone) and other bioactive substances such as nucleotide, peptide and amino acid, such as nicotinamide mononucleotide, nicotinamide adenine dinucleotide, L-alanine and L-glutamine (Sato et al., 1997).

EM-X has been shown to enhance the activities of the human T and B lymphocytes and natural killer cell, possess anti-HIV activity, increase the serum superoxide dismutase, decrease the malondialdehyde on the D-galactose-induced aging mice, possess antihyperglycemic effects and to prolong

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the life of fruit fly (Ke et al., 2001). EM-X inhibits the TNF- α and oxidant-induced interleukin-8 release in epithelial A543 cells and the peroxidation of phospholipids (Deiana et al., 2002) and modulates oxidative damage in the kidney and liver of rats by protecting unsaturated fatty acids (Aruoma et al., 2002). The ability of the antioxidant beverage to modulate neuronal cell death due to excitotoxicity has been documented (Aruoma et al., 2003). The loss of tyrosine hydroxylase positive cells in the substantia nigra pars compacta following the treatment of rats with the neurotoxin 6-hydroxydopamine has been shown to significantly attenuated by EM-X, further suggesting the neuroprotective potential of the beverage (Datla et al., 2004). The potential use of EM-X in the management of breast cancer patients has been suggested by Usmani et al. (2000). EM-X is widely available in Asia as a health beverage and there is the prospect of continued acceptance in clinical practice. Given this outcome, the aim of this study was to examine the acute, chronic and mutagenic toxicities of oral treatment of EM-X in animal models.

2. Materials and methods

2.1. Materials

NIH mice of both sexes weighing 20 ± 2 g were used in the acute toxicity test and those weighing 25–32 g were used for the mutagenic test. SD rats of both sexes weighing 110–130 g were used in the acute toxicity test. The animals were sex-separately housed in the air-conditioned animal rooms at temperature of 22 ± 2 °C and relative humidity of $60 \pm 5\%$. Foods and water were allowed ad libitum. In the mutagenic tests, the *S. Typhimurium*s of TA97, TA98, TA100 and TA102, which were carefully assayed to fit the demand gene types were used as testing strains in Ames tests. All testing strains were cultured over night and the bacterial concentrations were $\geq 10^{12}$ l⁻¹. S-9 was prepared from liver homogenate of rats induced by Aroclor 1254. The concentration of S-9 in the mixture that was composed of S-9 and assistant factors was 10% and S-9 protein contained in the mixture was 38 g/l. Dexon, sodium azide (NaN₃) and 2-aminofluorene (2AF) were used in Ames test as positive control agents and cyclophosphane was used in the test of marrow micronucleus and sperm shape assay. The original EM-X was obtained from EM Research Organization (Okinawa, Japan). Abbott cell-by-n (mode 1600, Abbott, America) and Hitachi chemistry analyzer (model 7170, Hitachi, Japan) were used in the chronic toxicity test.

2.2. Toxicity tests

Twenty mice were used in the acute toxicity test. EM-X was concentrated into a 20-fold liquid from the original EM-X. The mice were orally treated with the concentrate three times per day at the volume of 0.3 ml/10 g BW. The daily dose

was 90 ml/kg BW. Changes in body weight, food consumption pattern, behaviors and deaths were observed over 7 days. For the chronic toxicity test, 80 rats were equally divided into three EM-X dose groups of 180, 120 and 60 ml/kg and a control group. The rats of EM-X groups were orally treated with EM-X concentrates at the volume of 1.2 ml/kg once a day for 12 weeks. The EM-X concentrates were 150-, 100- and 50-fold of the daily dose used in clinic for the high-, medium- and low-dose groups, respectively. The control group was treated with the same volume of distilled water. The general habits of the states of the rats were observed daily. Changes in body weights were noted and the dosage of EM-X adjusted accordingly. Twenty-four hours after last administration of tested agents, 12 rats including both sexes in each group were killed and blood samples were collected. The hematological assays on red blood count (RBC), white blood-cell count (WBC), hemoglobin (Hb), blood platelets count (BPC), lymphocyte (L), granulocyte (GRAN), middle cell (MID) and coagulation time (CT) were performed using Abbott cell-by-n and the biochemical assays on the serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), total protein (TP), albumin (ALB), glucose (GLU), total bilirubin (T-BIL), creatinine (Crea) and total cholesterol (T-CHO) were performed using Hitachi chemistry analyzer. Physiological and histological examinations of the organs were carried out after dissection. The heart, liver, spleen, lung, kidney, renicapsule, thymus, testis, ovary and pancreas were removed, weighed and their organ coefficients assessed. The organs were then fixed with 10% formalin solution for 48 h for pathological examinations. Pathological sections were made with conventional methods and examined under light microscopy. The remaining rats were raised for a further 2 weeks at which point the administration of EM-X was stopped. The animals were sacrificed and the relevant tissues were used for the various assays.

In mutagenic test, EM-X was prepared into five concentrations of 5000, 500, 50, 5 and 0.2 μ g/disc with distilled water and treated with high-pressure sterilization for Ames tests. The stated dilutions of EM-X were used for the micronucleus test of bone marrow cell and sperm shape abnormality test on mice. Four testing strains, e.g. TA97, TA98, TA100 and TA102 of *S. Typhimurium* mutation types were used for Ames test. S-9 mixture was used for activating metabolic system in vitro. Growing bacterium solution of 0.1 ml and testing solution of 0.1 ml were added onto the upper layer of agar and S-9 mixtures were put on lower layer of culture medium plate. When metabolisms were activated S-9 mixture of 0.5 ml were added once again. Five doses of EM-X of 5000, 500, 50, 5 and 0.2 μ g/disc, spontaneous reversion, positive mutagen and non-bacterium control were designed in Ames test. The numbers of reverse colony in each disc were counted after cultivated for 48 h at 37 °C. When the rate of the increase in the reverse colony numbers exceeded that of the spontaneous reversion rate, the responses are considered positive if the effects are

dose dependent. Test was repeated twice under the same conditions.

For the micronucleus test of bone marrow cells, animals ($n = 60$) were divided into four groups of EM-X at doses of 5000, 2500, 1250 and 625 ml/kg BW, a normal control group (treated with the same volume of distilled water) and a positive control group (treated with cyclophosphane, 50 mg/kg BW). The mice were orally treated with EM-X of 0.4 ml/20 g BW twice a day for 5 days. The mice were killed 6 h after last administration. Immediately after, the breastbone marrows were removed and used for the preparation of smears after dilution with bovine serum albumin. The smears were fixed and the emerging colors observed under light microscopy. Polychromatophilic red cells (PRC) were counted in each mouse and micronucleus rate was expressed as thousandth rates of PRC.

For the sperm shape abnormality test mice ($n = 50$) were divided into three groups of EM-X at doses of 6000, 3000 and 1500 mg/kg BW, a normal control group (treated with the same volume of distilled water) and a positive control group (treated with cyclophosphane, 50 mg/kg BW). The mice were orally treated with EM-X of 20 ml/kg BW once a day for 5 days and were killed 30 days after the last EM-X administration of agents. The sperms of both epididymis were removed and use to prepare smears, which were fixed and analyzed using light microscopy. One thousand of complete sperm in each mouse were counted and the sperm shape abnormality rates were calculated.

2.3. Statistical analysis

The micronucleus test of bone marrow cell was analyzed with Poisson distribution. The sperm shape assay was analyzed with χ^2 -test. All data obtained from chronic toxicity are expressed as mean \pm S.D. and the mean differences between groups were considered to be significant at $P < 0.05$ with Student's *t*-test.

3. Results

It was not the purpose of this study to investigate the contribution of individual components in EM-X. The biological benefits of EM-X can be ascribed to the combined actions of the bioactive components and research is being directed at assessing their bioefficacy (Aruoma et al., 2002, 2003; Deiana et al., 2002).

3.1. Acute toxicity tests on EM-X

With treatment of 20-fold EM-X concentrate at daily dose of 90 ml/kg BW for 7 days, the food consumption, behaviors and stools of the mice tested were not altered. No toxic response was observed during 7 days of tests and there were no loss of animals. The mean body weight of the tested mice was increased by 28.2%. The maximal tolerance dose (MTD) of

original EM-X was calculated to be 1800 ml/kg BW and this corresponds to 1500 times of the daily dose (1.2 ml/kg BW) in clinic.

3.2. Chronic toxicity tests on EM-X

The general rat behavior, food consumption and stools both in the EM-X treated and control groups were normal, and there was no loss of animals during experiment. There were no abnormal changes in the parameters studied 2 weeks after stopping administration of EM-X. The mean body weight of the rats of medium dose group of EM-X was slightly increased at the 5th, 6th and 8th week as compared to the control group ($P < 0.05$). The changes in the body weights of the animals were not statistically significant and this was consistent with the report of Deiana et al. (2002). The principal organ coefficients of heart, liver, spleen, lung and kidney in the EM-X treated animal were not different from the control groups either at the 12 weeks of EM-X treatment or 2 weeks after the administration of EM-X was stopped (Table 1). The hematological assays on RBC, WBC, Hb, BPC, L, GRAN, MID and CT (Table 2) and the blood biochemical assays on AST, ALT, ALP, TP, BUN, ALB, GLU, T-BIL, Crea and T-CHO (Table 3) in the EM-X treated groups were similar to the control groups after 12 weeks of EM-X treatment and at 2 weeks after EM-X administration was stopped (this refers to "S" in the table of results). Pathological examinations showed that the tissue morphologies of principal organs of rats in all groups were normal. There were no significant pathological changes due to EM-X (Figs. 1–12). Hyperemia, swelling and necrosis were largely absent in the EM-X treated as well as the control animals.

3.3. Mutagenic tests on EM-X

The results of Ames tests are shown in Table 4. The reverse colony numbers of TA97, TA98, TA100 and TA102 in

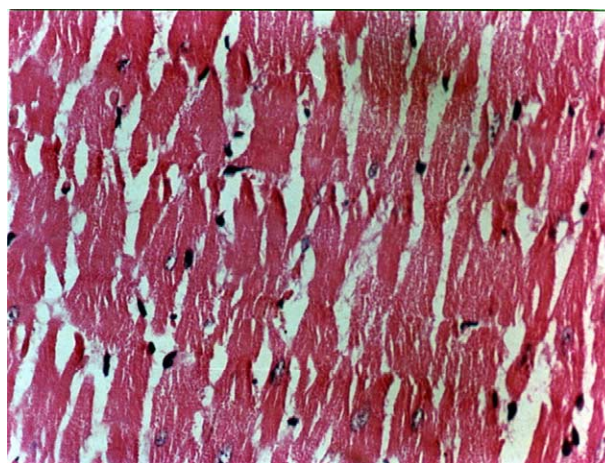


Fig. 1. Rats cardiac muscle photograph of control group. The tissue structure of cardiac muscle is normal. Muscle cells are clear and arranging in good order, no bleeding and necrosis (HE \times 650).

Table 1
Effects of EM-X on organ coefficient in rats

Organs	Groups							
	Control		Low dose of EM-X		Middle dose of EM-X		Higher dose of EM-X	
	D	S	D	S	D	S	D	S
Heart	0.31 ± 0.04	0.31 ± 0.04	0.34 ± 0.03	0.33 ± 0.04	0.34 ± 0.04	0.31 ± 0.04	0.31 ± 0.03	0.36 ± 0.04
Liver	3.36 ± 0.35	3.12 ± 0.34	3.60 ± 0.21	3.52 ± 0.38	3.31 ± 0.36	3.09 ± 0.29	3.36 ± 0.36	3.08 ± 0.53
Spleen	0.19 ± 0.04	0.21 ± 0.06	0.23 ± 0.03	0.21 ± 0.04	0.22 ± 0.03	0.20 ± 0.03	0.20 ± 0.02	0.25 ± 0.10
Lung	0.52 ± 0.09	0.51 ± 0.09	0.62 ± 0.09	0.53 ± 0.09	0.56 ± 0.09	0.51 ± 0.10	0.65 ± 0.18	0.60 ± 0.08
Kidney	0.66 ± 0.08	0.61 ± 0.07	0.69 ± 0.05	0.65 ± 0.07	0.65 ± 0.06	0.61 ± 0.05	0.62 ± 0.06	0.71 ± 0.09
Renicapsule (mg) ^a	18.52 ± 6.99	22.06 ± 6.02	18.96 ± 6.68	23.66 ± 5.72	18.54 ± 5.51	21.65 ± 7.76	19.12 ± 8.03	24.95 ± 5.23
Thymus	0.15 ± 0.04	0.16 ± 0.05	0.16 ± 0.03	0.18 ± 0.06	0.18 ± 0.04	0.17 ± 0.06	0.17 ± 0.04	0.20 ± 0.09
Testis	0.82 ± 0.02	0.76 ± 0.05	0.90 ± 0.11	0.86 ± 0.04	0.80 ± 0.09	0.84 ± 0.32	0.83 ± 0.12	0.88 ± 0.08
Ovary (mg)	52.23 ± 8.75	47.38 ± 3.61	53.08 ± 4.22	57.26 ± 8.76	57.58 ± 8.23	66.01 ± 6.24	52.07 ± 4.68	57.20 ± 11.19
Pancreas	0.24 ± 0.06	0.23 ± 0.05	0.21 ± 0.04	0.23 ± 0.04	0.23 ± 0.06	0.25 ± 0.08	0.23 ± 0.06	0.25 ± 0.06

D, treatment of agents for 12 weeks and S, 2 weeks after stop of agent treatment. No significant differences for all tests as compared to control.

^a Renicapsule refers to the adrenal gland.

Table 2
Effects of EM-X on hemological indexes in rats

Indexes	Groups							
	Control		Low dose of EM-X		Middle dose of EM-X		Higher dose of EM-X	
	D	S	D	S	D	S	D	S
RBC ($10^{12} l^{-1}$)	5.83 ± 0.67	5.67 ± 0.97	6.01 ± 0.40	6.15 ± 0.52	5.98 ± 0.41	6.19 ± 0.44	6.01 ± 0.40	6.35 ± 0.54
WBC ($10^9 l^{-1}$)	5.42 ± 1.12	6.52 ± 1.71	6.12 ± 2.46	5.97 ± 2.22	6.24 ± 1.82	5.18 ± 1.69	6.12 ± 2.46	5.40 ± 1.01
Hb (g/l)	124.3 ± 12.9	121.4 ± 18.6	125.3 ± 6.9	130.7 ± 9.0	128.7 ± 6.9	129.6 ± 5.5	125.3 ± 6.9	132.8 ± 8.9
BPC ($10^9 l^{-1}$)	581.8 ± 146.6	561.8 ± 123.7	670.7 ± 183.0	638.4 ± 240.2	681.1 ± 163.1	660.1 ± 124.2	670.7 ± 183.0	673.5 ± 78.6
L (%)	76.2 ± 6.6	74.8 ± 8.0	75.5 ± 5.9	76.6 ± 5.9	74.8 ± 6.9	79.3 ± 6.13	75.5 ± 5.9	78.1 ± 5.6
GRAN (%)	12.4 ± 4.5	12.9 ± 4.5	12.1 ± 3.9	11.7 ± 3.8	12.7 ± 4.8	9.09 ± 3.91	12.1 ± 3.9	9.96 ± 3.65
MID (%)	11.4 ± 2.7	12.2 ± 3.6	12.5 ± 2.4	11.8 ± 2.5	12.5 ± 2.6	11.5 ± 2.3	12.5 ± 2.4	11.9 ± 2.6
CT (min)	4.04 ± 0.78	4.14 ± 1.10	4.49 ± 2.46	4.32 ± 0.61	4.39 ± 0.65	4.28 ± 0.50	4.49 ± 0.43	3.96 ± 0.86

D, treatment of agents for 12 weeks and S, 2 weeks after stop of agent treatment. No significant differences for all tests as compared to control.

all dose groups of EM-X did not increase as compared to those of spontaneous reversion irrespective of the addition of the activating system of S-9. The micronucleus test of bone marrow cell show that micronucleus ratios in all dose groups of EM-X did not differ from those of normal control group; however, they were all significantly different from positive control group ($P < 0.01$ for all) (Table 5). The sperm shape abnormality ratio in all dose groups of EM-X did not show

any statistically significant difference ($P > 0.01$) when compared with the positive control group (Table 6).

4. Discussion

The cocktail EM-X is a beverage containing beneficial antioxidant compounds and has continued to arouse the interest

Table 3
Effects of EM-X on blood biochemical indexes in rats

Indexes	Groups							
	Control		Low dose of EM-X		Middle dose of EM-X		Higher dose of EM-X	
	D	S	D	S	D	S	D	S
AST (units)	172.8 ± 3.61	208.7 ± 36.2	150.3 ± 24.6	177.4 ± 22.1	160.2 ± 36.2	172.1 ± 30.4	155.1 ± 15.1	186.4 ± 34.6
ALT (units)	36.0 ± 6.3	33.7 ± 6.1	38.7 ± 9.3	37.1 ± 17.2	38.2 ± 14.7	35.6 ± 14.0	39.4 ± 11.0	30.1 ± 8.5
ALP (units)	285.2 ± 100.1	271.3 ± 68.8	303.2 ± 88.5	281.0 ± 69.9	243.0 ± 54.5	248.3 ± 40.4	301 ± 95.3	235.8 ± 95.4
BUN ($\mu\text{mol} l^{-1}$)	9.3 ± 1.5	7.71 ± 1.11	8.4 ± 0.9	9.3 ± 1.4	8.6 ± 1.2	7.8 ± 1.4	9.0 ± 0.9	8.2 ± 1.6
TP (g/l)	66.2 ± 3.9	66.6 ± 3.9	66.9 ± 3.6	69.6 ± 3.1	67.2 ± 2.2	68.3 ± 3.0	67.8 ± 4.0	66.7 ± 4.1
ALB (g/l)	34.9 ± 4.5	30.7 ± 5.7	32.6 ± 3.8	34.2 ± 2.9	33.2 ± 4.6	34.2 ± 4.6	33.4 ± 4.6	33.6 ± 5.6
GLU ($\text{mmol} l^{-1}$)	7.2 ± 1.1	6.8 ± 1.4	6.9 ± 1.1	6.1 ± 1.5	7.2 ± 0.8	5.9 ± 0.7	7.4 ± 0.8	5.4 ± 1.6
T-BIL ($\mu\text{mol} l^{-1}$)	1.1 ± 1.1	2.3 ± 1.1	1.1 ± 1.0	2.2 ± 0.5	1.0 ± 0.9	2.1 ± 0.6	1.2 ± 0.8	2.1 ± 1.2
Crea ($\mu\text{mol} l^{-1}$)	65.9 ± 13.7	73.6 ± 14.0	61.7 ± 8.9	70.6 ± 8.9	61.4 ± 6.6	67.3 ± 9.0	55.0 ± 6.7	72.5 ± 21.3
T-CHO ($\text{mmol} l^{-1}$)	1.5 ± 0.2	1.4 ± 0.2	1.4 ± 0.3	1.5 ± 0.3	1.6 ± 0.8	1.5 ± 0.2	1.5 ± 0.1	1.6 ± 0.2

D, treatment of agents for 12 weeks and S, 2 weeks after stop of agent treatment. No significant differences for all tests as compared to control.

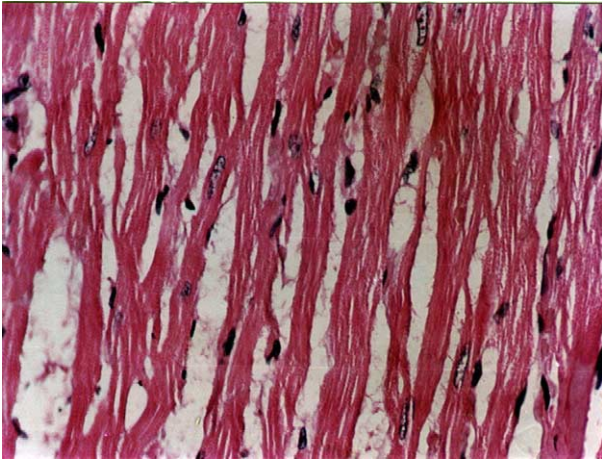


Fig. 2. Rats cardiac muscle photograph of EM-X higher dose group. The tissue structure of cardiac muscle is normal. Muscle cells are completely clearing, no bleeding and necrosis (HE \times 650).

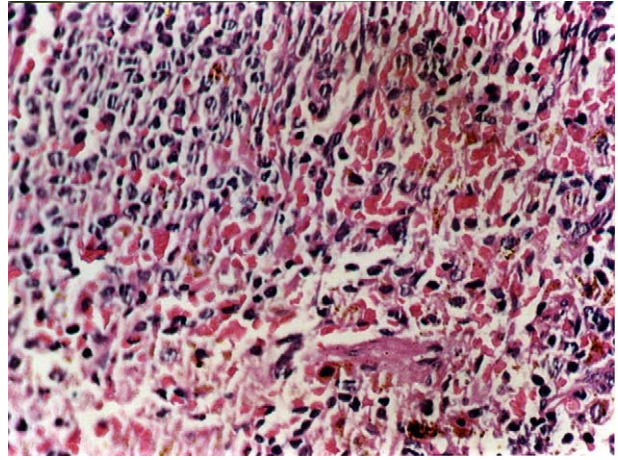


Fig. 5. Rat spleen photograph of control group. The tissue structure of spleen is normal. Spleen sinus did not show pathologic matter appearing (HE \times 650).

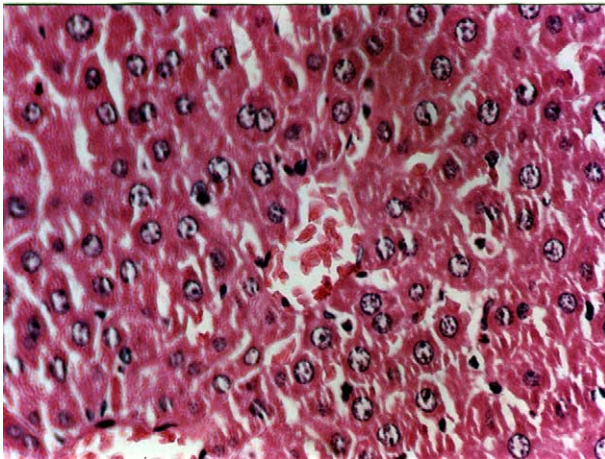


Fig. 3. Rat liver photograph of control group. The tissue structure of liver is normal, and no necrosis in hepatocyte. Hepato-rope, hepato-sinus and centre vein is clear, and hepato-rope show radiate arranging (HE \times 650).

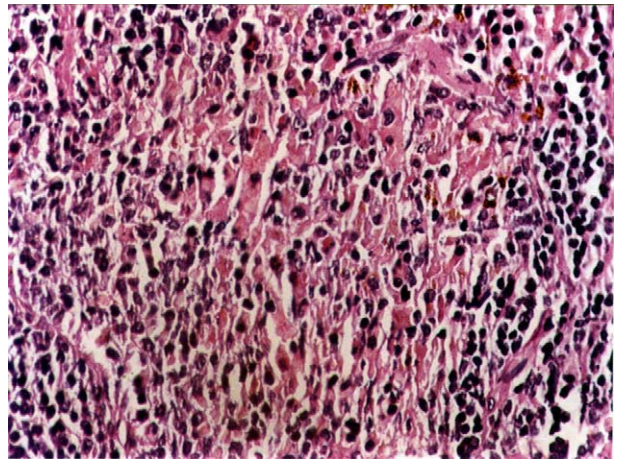


Fig. 6. Rat spleen photograph of EM-X higher dose group. The tissue structure of spleen is normal, and no block necrosis focus in spleen sinus (HE \times 650).

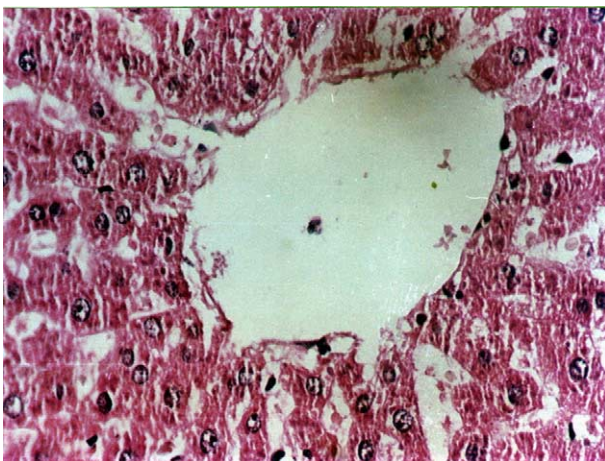


Fig. 4. Rat liver photograph of EM-X higher dose group. The tissue structure of liver is normal. The hepato-rope and hepato-sinus is very clearing and arranging in good order, no bleeding and necrosis focus (HE \times 650).

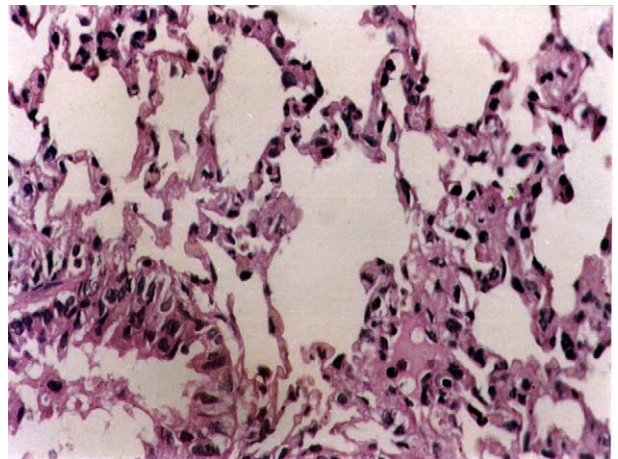


Fig. 7. Rat lung photograph of control group. The tissue structure of lung is normal. Epithelium of tiny bronchus mucosa is complete, and no foreign body in tube-cavity. The medium and cavity of pulmonary alveolus, blood vessel and epithelial cells morphology did not show abnormality (HE \times 650).

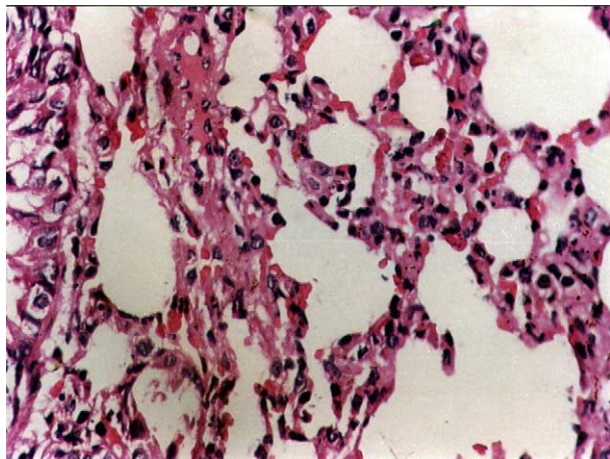


Fig. 8. Rat lung photograph of EM-X higher dose group. The tissue structure of lung is normal. The cavity of pulmonary alveolus shows dilatation, and wall of pulmonary alveolus did not show inflammatory reaction, no bleeding and necrosis (HE \times 650).

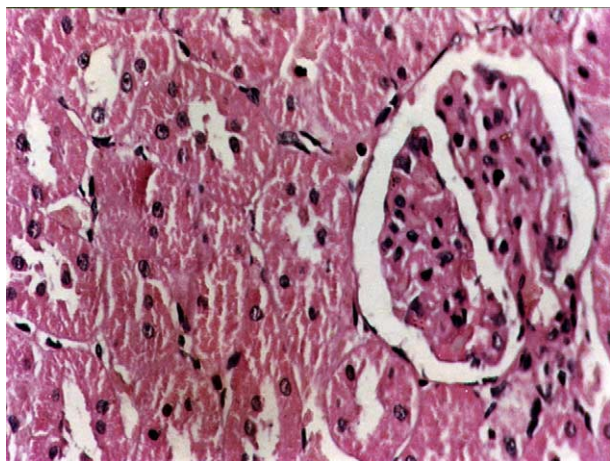


Fig. 9. Rat kidney photograph of control group. The tissue structure of kidney is normal. Both the glomeruli and tubular of kidney, and medium did not show abnormality (HE \times 650).

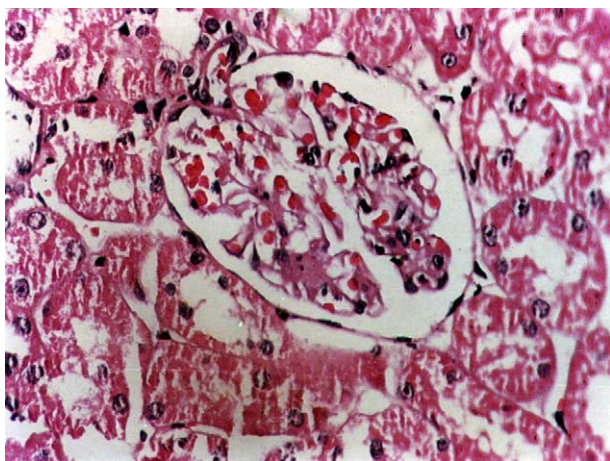


Fig. 10. Rat kidney photograph of EM-X higher dose group. The tissue structure of kidney is normal. The glomeruli and tubular of kidney may be seen clearly, no bleeding and necrosis (HE \times 650).

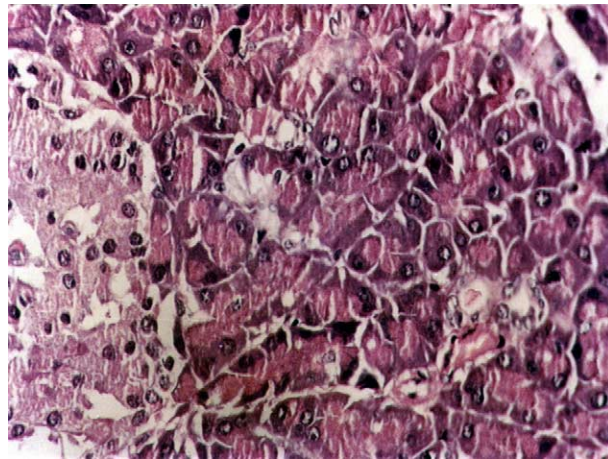


Fig. 11. Rat pancreas photograph of control group. Epithelial cells morphology in pancreas gland is normal. The each cells morphology in pancreas islet also did not show pathological change (HE \times 650).

of scientists and clinicians (Aruoma et al., 2002, 2003; Deiana et al., 2002; Ke et al., 2001; Usmani et al., 2000). Since EM-X is consumed as presented, we undertook this study to examine its acute, chronic and mutagenic toxicities in experimental animals. EM-X dose in the acute toxicity study reached 1800 ml/kg BW. This level corresponded to 1500 times of the daily dose in clinic. No toxic response was observed, indicating the safety of oral administration of EM-X. The contribution of each of the components of EM-X was not examined in this study. However, future research and development on the beverage involves optimization of the flavonoid and saponin contents.

For the 12-week chronic toxicity study, EM-X was used at doses of 60, 120 and 180 ml/kg, which were equivalent to 50, 100 and 150 times of the daily doses in clinic, respectively. This treatment did not affect the general animal states and the principal organ coefficients. The hematological and biochemical parameters were not significantly different.

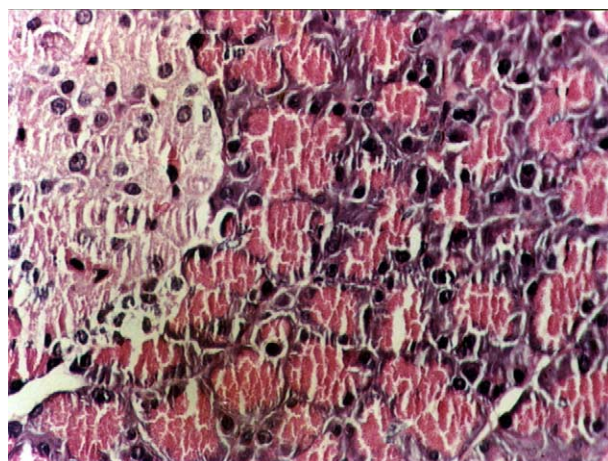


Fig. 12. Rat pancreas photograph of EM-X higher dose group. The tissue structure of pancreas is normal, and did not show other pathologic change (HE \times 650).

Table 4
Effects of EM-X on reverse colony numbers in Ames strains

Dose ($\mu\text{g}/\text{disc}$)	TA97		TA98		TA100		TA102	
	–(S-9)	+(S-9)	–(S-9)	+(S-9)	–(S-9)	+(S-9)	–(S-9)	+(S-9)
5000	149	159	47	48	157	160	307	321
500	147	154	47	47	159	160	284	318
50	145	150	48	47	148	156	283	316
5	147	153	44	46	153	152	305	311
0.2	146	155	44	45	153	358	308	315
Spontaneous reversion	141	155	46	44	145	158	311	315
Positive control								
–(S-9)								
Dexon (50 $\mu\text{g}/\text{disc}$)		TA97		2552				
Dexon (50 $\mu\text{g}/\text{disc}$)		TA98		1092				
NaN_3 (1.5 $\mu\text{g}/\text{disc}$)		TA100		2707				
Dexon (50 $\mu\text{g}/\text{disc}$)		TA102		935				
+(S-9)								
2AF (10 $\mu\text{g}/\text{disc}$)		TA97		1672				
2AF (10 $\mu\text{g}/\text{disc}$)		TA98		5808				
2AF (10 $\mu\text{g}/\text{disc}$)		TA100		2998				
2AF (10 $\mu\text{g}/\text{disc}$)		TA102		602				

The results are means of three disc. No significant differences for all tests as compared to the control.

Table 5
Effects of EM-X on micronucleus rate of PRC in mice marrow

Dose (mg/kg BW)	No. of animal		No. of cells observed		No. of MN cells observed		Rate of MN	
	♂	♀	♂	♀	♂	♀	♂	♀
5000	5	5	5000	5000	6	6	1.2	1.2
2500	5	5	5000	5000	5	6	1.0	1.2
1250	5	5	5000	5000	5	6	1.0	1.2
625	5	5	5000	5000	6	6	1.2	1.2
Normal control	5	5	5000	5000	7	6	1.4	1.2
Positive control	5	5	5000	5000	122	117	24.4*	23.4*

* $P < 0.01$ compared to all doses of EM-X and normal control.

The morphologies of the principal organs were normal when compared with the controls. There was no delayed reaction 2 weeks after the administration of EM-X was stopped. The data suggest that long-term oral administration of EM-X at daily dose is safe. In the Ames tests, the S-9 mixtures worked well when added into culture medium. However, the reverse colony numbers in all testing strains did not increase in all dose groups of EM-X as compared to the control. The results suggest that EM-X did not cause mutagenicity of cells. Results of micronucleus ratios of bone marrow cell of mice in all dose groups of EM-X were quite similar to normal

controls and significantly differed from the positive controls, indicating that treatment of EM-X did not induce numerical and structural chromosomal damage in the cells. Moreover, sperm shape abnormality rate in all dose groups of EM-X also did not show any difference from the normal controls. The results showed that EM-X had no influence on the micronucleus ratio and on the sperm shape abnormality upon EM-X treatment. Taken together, we conclude that oral administration of EM-X does not result in any toxic complications resulting from either acute or chronic use.

Table 6
Effects of EM-X on malformed sperm rate in mice

Dose (mg/kg BW)	No. of animal	No. of sperms observed	No. of malformed sperms observed	Malformed sperm rate of mean (%)
6000	8	8000	213	2.66
3000	8	8000	212	2.65
1500	8	8000	208	2.60
Normal control	10	10000	277	2.77
Positive control	6	6000	534	8.9*

* $P < 0.01$ compared to all doses of EM-X and normal control.

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