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GENOTOXICITY AND CARCINOGENICITY

The inhibitory effects of garlic and *Panax ginseng* extract standardized with ginsenoside Rg3 on the genotoxicity, biochemical, and histological changes induced by ethylenediaminetetraacetic acid in male rats

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Abstract Ethylenediaminetetraacetic acid (EDTA) is widely used in food and other industries to sequester metal ions and to prevent their disadvantageous effects. The objective of the current study was to evaluate the protective effect of *Panax ginseng* extract standardized with ginsenoside Rg3 (ginsenoside Rg3 content was 3.6% w/w, i.e., 36 µg/mg *P. ginseng* extract) and garlic against EDTA-induced biochemical, genotoxic, and histological changes in rats. Forty male rats were divided into eight treatment groups and treated for 7 days as follows: the control group, the group treated with EDTA (20 mg/kg b.w) and the groups treated with *P. ginseng* extract (20 mg/kg b.w), garlic (5 mg/kg b.w), *P. ginseng* plus garlic alone or in combination with EDTA. In vivo bone marrow micronucleus test and random amplified polymorphism DNA-PCR (RAPD-PCR) method were performed to assess the antigenotoxic effect of both protective agents. The results indicated that EDTA administration caused a significant decrease in the serum biochemical parameters and antioxidant enzymes activity. The administration also increased lipid peroxidation and the incidence of micronucleated polychromatic erythrocytes (MnPCEs), caused appearance of some changes in polymorphism band patterns, and induced different histopathological lesions in the livers, kidneys, and testis. Treatment with *P. ginseng*, garlic alone or plus EDTA significantly improved all the tested parameters. Moreover, *P. ginseng* extract was found to be more effective than garlic in restoring the parameters that were altered by EDTA.

Keywords Ethylenediaminetetraacetic acid · *Panax ginseng* · Garlic · Genotoxicity · Protection

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Introduction

Ethylenediaminetetraacetic acid (EDTA) is one of the most important chelating agents that widely used in food and other industries to sequester metal ions and to prevent their disadvantageous effects (Fishbein et al. 1970; Heindorff et al. 1983; Muralidhara and Narasimhamurthy 1991). FDA has also approved EDTA as a food additive that is generally recognized as safe (GRAS). EDTA's array of biochemical properties make it extremely valuable as a food additive. In pharmacy and medicine, EDTA has become a valuable drug for the regulation of metal-ion concentrations in biological systems, as well as for the removal of noxious substances from the human body (Fishbein et al. 1970; Heindorff et al. 1983).

Previous reports indicated that EDTA induced DNA damage or chromosome breaking in human (Basrur and Baker 1963) and mice (Manna and Das 1976). Chromosome

aberrations are induced in bone marrow cells and spleen cells in vivo of mice after injection of EDTA (Heindorff et al. 1983). EDTA also induces UDS in SHE cells (Fukuda 1987). However, it does not induce morphological transformation in the same cells (Fukuda 1987). Hagiwara et al. (2006) reported increases in the levels of chromosome aberrations were induced in SHE cells treated with EDTA. Because EDTA is a chelating agent, the sequestering of metal ions by EDTA may be responsible for the aberrations of the genetic apparatus. However, the molecular mechanisms leading to such potentiation are not clearly understood. The wide use of EDTA and its salts as food additives, especially in human diets including breakfast cereals and cereal bars (Heimbach et al. 2000), its known calstogenic potency and its ability to potentiate the mutagenic response of chemical and physical agents clearly necessitates the generation of comprehensive data on its mutagenic potency in well validated in vivo mammalian systems.

There is growing evidence in the literature to use some plant extracts that possess an array of interesting pharmacological effects. *Ginseng* is the traditional herbal remedy used in Chinese medicine for thousands years (Loo et al. 2004). One of the most commonly used and researched *ginsengs* is *Panax ginseng*, also called Asian or Korean *ginseng* (Kakizoe 2000). The main active components of *P. ginseng* are ginsenosides, which have been shown to have a variety of beneficial effects, including anti-inflammatory, antioxidant, and anticancer effects (Lee et al. 1998; Kakizoe 2000; Kampen et al. 2003; Loo et al. 2004).

Garlic (*Allium sativum*) is a common spicy flavoring agent used since ancient times. It has been shown to posses many medicinal properties including bactericidal, hypolipidemic, hypocholesteromic, antineoplastic and anticancer effects (Jonkers et al. 1999; Duncan 1999; Siegers et al. 1999) and antioxidant properties (Abdel-Wahhab and Aly 2003, 2005; Abdel-Wahhab et al. 2004). Moreover, garlic has chemopreventive potential against cyclophosphamide induced chromosomal mutations in Swiss albino mice (Shukla and Taneja 2002). The aims of the current study are twofold (1) to evaluate the potency of EDTA to induce biochemical changes, bone marrow micronuclei, DNA damage, and histological alterations in rats, and (2) the ability of *P. ginseng* extract and garlic to prevent the toxic effects of EDTA.

Materials and methods

Chemicals and kits

Ethylenediaminetetraacetic (CAS no. 60-00-4) was purchased from Sigma, St. Louis, MO, USA. Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST), Lactate dehydrogenase (LDH), albumin, createnine, and blood urea nitrogen (BUN) kits were obtained from BioMe´rieux, Laboratory of Reagents and Products, Marcy Létoile, France. MDA kit was purchased from Oxos Research™ Co., kit (USA). Glutathione peroxidase (GPX) and Superoxide dismutase (SOD) kits were purchased from Randox Laboratories Co., Crumlin, UK.

Plant extracts

P. ginseng

The standardized *P. ginseng* extract EFLA400 (*Phoenix ginseng*) (Batch no. 303298) of *P. ginseng* C. A. Mayer was prepared according to the published procedure (Korean patent 0425022, PCT/KR2003/000003) and was supplied from Lotte Group R & D Center, Seoul, South Korea. The content of ginsenoside Rg3, a pharmacologically active ingredient of phoenix *ginseng*, was 3.6% (w/w) as determined by HPLC (i.e., 36 μg/mg *p. ginseng* extract).

Preparation of garlic

Fresh garlic bulbs were purchased from the local market, Cairo, Egypt, homogenized and freeze dried using Freeze dryer system (Dura-Dry Freeze Dryer, Model PAC-TC-V4; FTS system Inc., Stone Ridge, NY, USA). The dried garlic powder was kept at −40°C until used.

Experimental animals

Forty adult male albino rats (100-130 g, purchased from the Animal House Colony, Giza, Egypt) were maintained on standard laboratory diet (protein, 16.04%; fat, 3.63%; fiber, 4.1%; and metabolic energy, 0.012 MJ) and water ad libitum at the Animal House Laboratory, National Research Center, Dokki, Cairo, Egypt. After an acclimation period of 1 week, animals were divided into eight groups (5 rats/group) and housed individually in filter-top polycarbonate cages housed in a temperature-controlled (23 ± 1°C) and artificially illuminated (12 h dark/light cycle) room free from any source of chemical contamination. All animals received humane care in compliance with the guidelines of the Animal Care and Use Committee of National Research Center, Egypt.

Experimental design

Animals within different treatment groups (Table 1) were treated (daily at a 24-h interval) intragastrically for 1 week as follows: group 1, untreated control; group 2, treated intragastrically with 5 mg/kg b.w. of garlic powder

Table 1 Experimental groups and the respected treatments ($n = 5$)

Groups	Treatment	Dose (mg/kg b.w)
1	Untreated control	–
2	Garlic	5
3	*P. ginseng*	20
4	Garlic + *P. ginseng*	5 + 200
5	EDTA (positive control)	200
6	Garlic + EDTA	5 + 200
7	*P. ginseng* + EDTA	20 + 200
8	Garlic + *P. ginseng* + EDTA	5 + 20 + 200

suspended in distilled water; group 3, treated with 20 mg/kg b.w. of *P. ginseng* extract dissolved in distilled water; group 4, treated with 5 mg/kg b.w. of garlic and 20 mg *P. ginseng*; group 5, treated with 200 mg/kg b.w. of EDTA (i.e., 1/10 LD_{50}) suspended in distilled water (positive control group); group 6, treated with garlic plus EDTA; group 7, treated with *P. ginseng* plus EDTA and group 8, treated with garlic and *P. ginseng* plus EDTA. At the end of the experimental period, blood samples were collected from the retro-orbital venous plexus of all animals after being fasting for 12 h for DNA isolation and the biochemical analysis. The activities of ALT, AST, and BUN were determined according to the method recommended by Henry et al. (1974). Serum albumin was determined according to Doumas et al. (1971), createnine was determined according to Bartles et al. (1972), and LDH was determined according to Tietz (1987). All animals were sacrificed and dissected on day 8. Bone marrow samples were collected from both femurs of each animal and extracted immediately and processed for the MN assay.

Biochemical analysis

Livers, kidneys and testes were collected for the histopathological examinations. Another sample of liver of each animal was dissected, weighed and divided into two portions. The first portion was homogenized in phosphate buffer (pH 7.4) to give 20% w/v homogenate (Lin et al. 1998). This homogenate was centrifuged at 1,700 rpm and 4°C for 10 min and the supernatants was stored at −70°C to the next day until analysis. This supernatant (20%) was used for the determination of hepatic lipid peroxidation (LPO) and it was further diluted with phosphate buffer solution to give 2 and 0.5% dilutions for the determination of hepatic GPX (2%) and SOD (0.5%) activities. Hepatic LPO was estimated by the measurement of malondialdehyde (MDA) by spectrophotometeric method (Esterbauger et al. 1991). The level of lipid peroxides was expressed as μmol MDA/mg protein. The protein content in liver tissue was measured by applying the method of Lowry et al. (1951). Hepatic GPX activity was determined by spectrophotemetric method using reduced glutathione and cumene hydroperoxide as substrate using 20 μl diluted liver homogenate according to the modified method of Paglia and Valentine (1967). Hepatic SOD activity was assayed spectrophometerically by red formazan dye reduction procedure (Suttle 1986) using 50 μl diluted liver homogenate. The specific activity of hepatic GPX and SOD was expressed as unit/mg liver protein.

MN assay

The bone marrow cells resuspended in a small volume of fetal calf serum on a glass slide were used for smear preparation. The smear of bone marrow cells was prepared from each rat. After air-drying, the slide was fixed in methyl alcohol for 10 min and stained with 5% Giemsa stain for 10 min. Three slides were prepared for each animal and were coded before observation and one was selected for scoring. From each coded slide, 3,000 polychromatic erythrocytes (PCEs) were scored for the presence or micronuclei under oil immersion at high power magnification. In addition, the percentage of micronucleated polychromatic erythrocytes (%MnPCEs) was calculated on the basis of the ratio of MnPCEs to PCEs (Adler 1984).

Molecular analysis

The genomic DNA was isolated using phenol/chloroform extraction and ethanol precipitation method with minor modifications (Sambrook et al. 1989). The purity of the DNA was evaluated by absorbances at 230, 260, and 280 nm (Aquardo et al. 1992). When impurities were present (pure DNA has a ratio $A_{260}/A_{230} = 1.7–2.2$), the DNA sample was passed through the commercial UltraClean Soil kit until satisfactory purity was reached.

RAPD-PCR analysis

To generate RAPD profiles from rats DNA, five oligodecamers (5-mer random primers: A01: 5′-CAGGCCCTTC-3′, A02: 5′-TGCCGAGCTG-3′, A10: 5′-GTGATCGCAG-3′ and C09: 5′-CTCACCGTCC-3′, C15: 5′-GACGGATCAG-3′) from Operon Technologies, Almeda, CA, USA, were used. DNA amplification reactions were performed under conditions reported by Luceri et al. (2000). PCR amplification was conducted in 50 μl reaction volume containing 100 ng genomic DNA; 100 μM dNTPs; 40 nM primer (Operon); 2.5 U of Taq DNA polymearse and 5 μl promega 10X Taq DNA polymearse buffer. The reactions were carried out in Thermocycler (Perkin-Elmer 9700) programmed with a first denaturation of 5 min at 94°C, followed by 45 cycles of 0.5 min at 94°C, 1 min at 36°C and 2 min at 72°C and finally, one cycle at 72°C for 5 min. The

PCR product was analyzed by electrophoresing 25 μl of the amplified mixture on agarose gel. The Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD, USA) was used to document ethidium bromide DNA gels.

Histopathological examination

Tissue samples (liver, kidneys, and testes) were fixed in neural buffered formalin 10%, dehydrated in ascending concentrations of ethanol, cleaned in xylene, and embedded in paraffin. Sections 4–5 μm thick were prepared and stained with hematoxylin and eosin (Bancroft et al. 1996).

Statistical analysis

The binomial data for biochemical analysis and micronucleus showed normal distribution. Therefore, all data were analyzed using the General Liner Models (GLM) procedure of Statistical Analysis System (SAS 1982) followed by Scheffé-test to assess significant differences between groups. The values are expressed as mean ± SEM. All statements of significant were based on probability of $P < 0.05$.

Results

The acute toxicity of EDTA first appeared as a significant decrease in food intake accompanied by a decrease in body weight gain (data not shown). The animal in this group continued to refuse the feed to reach nothing on day by the seventh day of treatment. Whereas, all animals in the other groups continued to consume feed and gain normal weight. By the end of day 7 we stopped the experiment due to the severe toxicity for the animals in EDTA group.

Biochemical assay

Results of the biochemical study revealed that treatment with EDTA alone caused a significant decrease in ALT, AST, LDH, albumin, createnine, and BUN, whereas, no significant differences were noticed in the animals treated with *P. ginseng* alone, garlic alone or *P. ginseng* plus garlic. Animals treated with EDTA plus *P. ginseng*, EDTA plus garlic, or EDTA plus *P. ginseng* in combination with garlic showed a significant improvement in all the biochemical parameters tested. Although *P. ginseng* extract was found to be more effective than garlic in this respect. Moreover, the combined treatment of *P. ginseng* plus garlic was the most effective one against the biochemical changes induced by EDTA compared to the individual treatment of *P. ginseng* or garlic alone (Table 2).

The effects of different treatments on the oxidant–antioxidant status of the livers are depicted in Table 3. It is worthy

Table 2 Effect of EDTA alone or in combination with garlic, *P. ginseng* and garlic plus *P. ginseng* on serum biochemical parameters in rats

Groups	Treatment							
	Control	Gr.	Gn.	Gn. + Gr.	EDTA	EDTA + Gr.	EDTA + Gn.	EDTA + Gn. + Gr.
ALT (IU/l)	30.51 ± 2.16^a	30.51 ± 2.28^a	29.27 ± 1.35^a	30.55 ± 2.82^a	22.55 ± 3.18^b	26.11 ± 2.34^c	27.16 ± 1.72^c	29.53 ± 2.16^a
AST (IU/l)	35.82 ± 3.13^a	36.78 ± 2.91^a	34.95 ± 2.74^a	36.88 ± 2.36^a	29.11 ± 2.47^b	33.13 ± 1.21^a	33.44 ± 1.65^a	36.53 ± 1.57^a
LDH (μ/l)	177.35 ± 9.36^a	175.69 ± 8.32^a	181.52 ± 6.11^a	185.67 ± 5.34^b	150.32 ± 2.51^c	172.53 ± 4.22^a	179.32 ± 5.31^a	180.16 ± 4.54^a
Albumin (g/dl)	4.15 ± 0.22^a	4.56 ± 0.31^a	3.99 ± 0.33^a	5.07 ± 0.41^b	2.46 ± 0.17^c	3.99 ± 0.41^a	4.22 ± 0.16^a	4.58 ± 0.98^a
Createnine (mg/dl)	0.82 ± 0.06^a	0.79 ± 0.07^a	0.93 ± 0.08^b	0.98 ± 0.09^b	0.66 ± 0.04^c	0.79 ± 0.08^a	0.80 ± 0.03^a	0.86 ± 0.19^a
BUN (mg/dl)	24.23 ± 1.16^a	25.17 ± 1.09^a	25.09 ± 1.05^a	26.13 ± 1.07^b	19.15 ± 1.32^c	22.15 ± 1.09^d	24.17 ± 2.36^a	25.77 ± 1.82^a

Within each column, means with the same superscript are not significantly different ($P \leq 0.05$)

Gn P. ginseng, *Gr* garlic

Table 3 Effect of garlic and *P. ginseng* on the oxidant–antioxidant status of liver in EDTA-treated rats (mean + SE)

Groups	Parameters		
	Lipid peroxidation (μmol/mg liver protein)	GPX (U/mg liver protein)	SOD (U/mg liver protein)
Control	35.4 ± 1.6^{a}	1.87 ± 0.04^{a}	103.2 ± 2.4^{a}
Ginseng	36.2 ± 1.5^{a}	1.95 ± 0.13^{a}	104.4 ± 1.4^{a}
Garlic	34.6 ± 1.8^{a}	1.98 ± 0.07^{a}	117.6 ± 3.2^{b}
Ginseng + Garlic	36.8 ± 2.7^{a}	1.99 ± 0.03^{a}	123.7 ± 3.8^{c}
EDTA	66.4 ± 2.6^{b}	0.62 ± 0.02^{b}	74.8 ± 2.9^{d}
EDTA + *Ginseng*	42.3 ± 2.3^{c}	0.92 ± 0.05^{c}	98.9 ± 5.3^{e}
EDTA + Garlic	43.4 ± 1.9^{c}	0.99 ± 0.08^{c}	99.6 ± 2.7^{e}
EDTA + *Ginseng* + Garlic	38.2 ± 1.7^{a}	1.57 ± 0.03^{d}	102.4 ± 3.6^{a}

Within each columns, means superscript with the same letter are not significantly different ($P > 0.05$)

GPX glutathione peroxidase, *SOD* superoxide dismutase

to mention that EDTA produced a significant increase in hepatic LPO level with concomitant significant decrease in hepatic GPX and SOD. Animals treated with *P. ginseng* alone were comparable to the control regarding the level of LPO or the activity of GPX and SOD. Treatment with garlic alone or garlic plus *P. ginseng* resulted in a significant increase in SOD whereas MDA and GPX were in the normal level of the control. The combined treatment with *P. ginseng* or garlic plus EDTA, as well as the treatment with garlic and *P. ginseng* plus EDTA led to a significant improvement in the oxidant–antioxidant status of the liver of the treated rats compared with those treated with EDTA alone. This improvement was pronounced in the animals treated with the combination of *P. ginseng* and garlic plus EDTA since this group was comparable to the control group (Table 3).

MN assay

Data in Table 4 shows the effects of different treatments on the bone marrow MnPCEs. The results revealed that EDTA was able to cause a high incidence of MnPCEs in rats (Fig. 1). The number of MN in polychromatic erythrocytes in the bone marrow cells after EDTA administration increased significantly compared with the values of control. *P. ginseng* extract alone or in combination with garlic were able to reduce the elevation of MnPCEs number in the bone marrow cells resulted from EDTA administration. This inhibition in MnPCEs reached 51 and 42% in the groups treated with *P. ginseng* extract alone *or P. ginseng* plus garlic, respectively. Meanwhile, garlic alone was not significantly efficient to decrease the MN formation in the bone marrow induced by EDTA (28%).

RAPD fingerprinting pattern

The molecular genetic variability among the treated rat genomes was evaluated using five oligonucleotide primers (A01, A02, A10, C09, and C15). Two primers (A10 and

Table 4 Inhibitory effects of ginseng and garlic alone and in combination on EDTA- induced bone marrow MnPCEs

Groups	Parameters		
	MnPCEs (%)	MnPCEs/2,000 PCEs	
		Mean	SEM
Control	0.36	7.2^{c}	0.58
Ginseng	0.41	8.2^{bc}	0.37
Garlic	0.41	8.2^{bc}	0.58
Ginseng + Garlic	0.40	8.0^{bc}	0.89
EDTA	0.85	17.0^{a}	0.84
EDTA + *Ginseng*	0.40	8.2^{bc}	0.37
EDTA + Garlic	0.61	12.2^{ab}	1.02
EDTA + *Ginseng* + Garlic	0.49	9.8^{bc}	1.46

PCEs polychromatic erythrocytes, *MnPCEs* micronucleated polychromatic erythrocytes, within each column means superscripts with different letters are significantly different ($P \leq 0.05$), total counted PCEs was 15,000 per a group

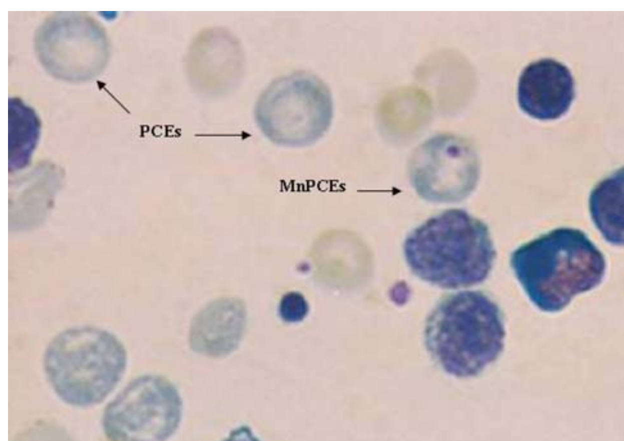

Fig. 1 Normal polychromatic erythrocytes (*PCEs*) and micronucleated polychromatic erythrocytes (*MnPCEs*) in male rats treated with EDTA

C09) only gave positive and detectable bands (Fig. 2), where, they amplified a total of 57 different bands, ranging from 280 to 1,516 bp (Table 5). Of the 57 scorable bands, one was similar "monomorphic" for control, *P. ginseng*, garlic and *P. ginseng* plus garlic (A10-530); six were similar for control, *P. ginseng* or garlic (A10-1163, A10-795, A10-714, A10-603, A10-304, and, C09-299); three were similar for control and *P. ginseng* (C09-708, C09-488, and, C09-383); and four were similar for garlic or garlic plus *P. ginseng* (C09-889, C09-686, C09-446, and, C09-372) (Table 5). However, only two bands (A10-1328 and A10-946) were monomorphic for control, *P. ginseng*, garlic and EDTA samples (Table 5).

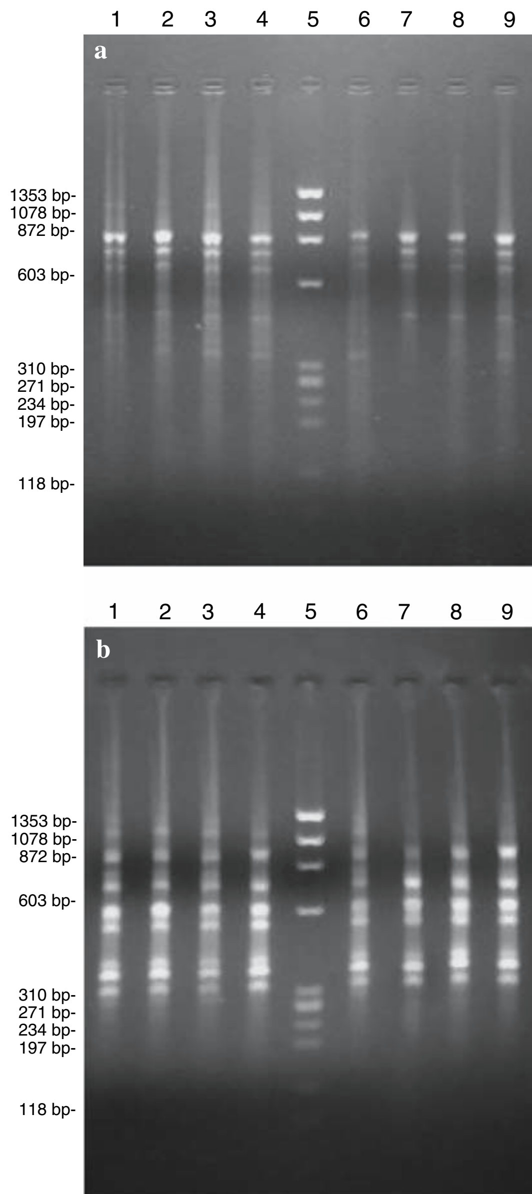

Fig. 2 Comparison of RAPD fingerprinting profiles of different rat genomic DNA. **a** Represents PCR products with primer A10, **b** represents PCR products with primer C09. The DNA marker was in lane 5. Lane 1 represents untreated rats, lane 2 represents rats treated with *P. ginseng*, lane 3 represents rats supplemented with garlic, lane 4 represents rats treated with *P. ginseng* plus garlic, lane 6 represents rats exposed to EDTA alone, Lane 7 represents rats treated with *P. ginseng* plus EDTA, Lane 8 represents rats treated with garlic plus EDTA, Lane 9 represents rats administrated with *P. ginseng* in combination with garlic plus EDTA

The DNA of the samples treated with EDTA analyzed with the two primers revealed the appearance of eight new bands (A10-1516, A10-852, A10-736, A10-631, A10-415, A10-377, A10-325, and, C09-281) which did not appear in the samples of other treatment groups (Table 5). These new bands could be considered as potential markers which attributed to EDTA treatment. Furthermore, RAPD primer C09 in the samples of rat exposed to EDTA displayed loss of 18 stable bands (C09-906, C09-889, C09-779, C09-767, C09-708, C09-686, C09-675, C09-488, C09-459, C09-446, C09-383, C09-372, C09-361, C09-299, C09-297, C09-285, C09-283, and, C09-280) which occurred in the DNA of normal or protected rat samples (Fig. 1).

Histopathological results

The histopathological examination of liver tissues of rat treated with EDTA alone showed kupffer cells activation, sporadic cell necrosis, dilated hepatic sinusoids as well as massive vacuolations of periportal hepatocytes (Fig. 3a, b). Multifocal areas of hepatocytic necrosis associated with mononuclear leucocytic cells infiltration were also observed in this group (Fig. 3c). Liver of rats treated with garlic or *P. ginseng* extract showed normal histopathological pictures. However, sections of liver from rats treated with EDTA plus garlic revealed activation of kupffer cells, hyperplasia of epithelial lining bile ducts associated with oedema in the portal tracts (Fig. 3d). Moreover, liver of rat treated with EDTA plus either *P. ginseng* extract alone or with *P. ginseng* extract plus garlic revealed no histopathological changes except proliferation of kupffer cells (Fig. 3e, f).

Histopathological examination of the kidney tissues in EDTA-treated rat revealed marked vacuolar degeneration of epithelial lining proximal convoluted renal tubules as well as endothelial lining the glomerular tufts associated with hypertrophy of the tufts with narrowing of Bowman's spaces (Fig. 4a, b). Small focal area of tubular necrosis replaced by mononuclear leucocytic cells was noticed in some sections (Fig. 4c). Kidney of garlic or *P. ginseng* extract-treated rats showed normal histopathological pictures. The sections from rats treated with EDTA plus either *P. ginseng* extract or garlic revealed no histopathological changes except congestion of renal blood vessels (Fig. 4d, e). Apparent normal histological picture was observed in kidney sections of rat treated with EDTA combined with garlic plus *P. ginseng* extract (Fig. 4f).

Table 5 Size in base pair of detected rats markers

	Control	Gn	Gr	Gn + Gr	EDTA	EDTA + Gn	EDTA + Gr	EDTA + Gn + Gr		Control	Gn	Gr	Gn + Gr	EDTA	EDTA + Gn	EDTA + Gr	EDTA + Gn + Gr		Control	Gn	Gr	Gn + Gr	EDTA	EDTA + Gn	EDTA + Gr	EDTA + Gn + Gr
A10-516					+				A10-03	+	+	+						C09-792	+	+			+	+		
A10-1328	+	+	+		+				A10-584						+	+	+	C09-779				+			+	+
A10-1278				+					A10-530	+	+	+	+					C09-767			+					
A10-1231						+	+	+	A10-436							+	+	C09-708	+	+						
A10-1163	+	+	+						A10-422						+			C09-697					+		+	
A10-1141				+	+				A10-415					+				C09-686			+	+				
A10-962						+	+	+	A10-402				+					C09-675						+		+
A10-946	+	+	+		+				A10-389						+			C09-488	+	+						
A10-931				+					A10-383	+	+	+				+	+	C09-459								+
A10-821								+	A10-377					+				C09-446			+	+				
A10-825					+				A10-365									C09-383	+	+						
A10-795	+	+	+						A10-347	+	+	+				+	+	C09-372			+	+				
A10-771						+	+		A10-336						+			C09-361						+	+	+
A10-748				+					A10-320				+					C09-299	+	+	+					
A10-736					+				A10-325					+				C09-297				+				
A10-725				+					A10-304	+	+	+						C09-285		+	+					
A10-714	+	+	+						C09-926					+	+	+		C09-283	+							
A10-651						+	+		C09-906	+	+						+	C09-281					+			
A10-631					+			+	C09-889			+	+					C09-280				+				

(+) each marker was found in control and treated samples

Gn ginseng, Gr garlic

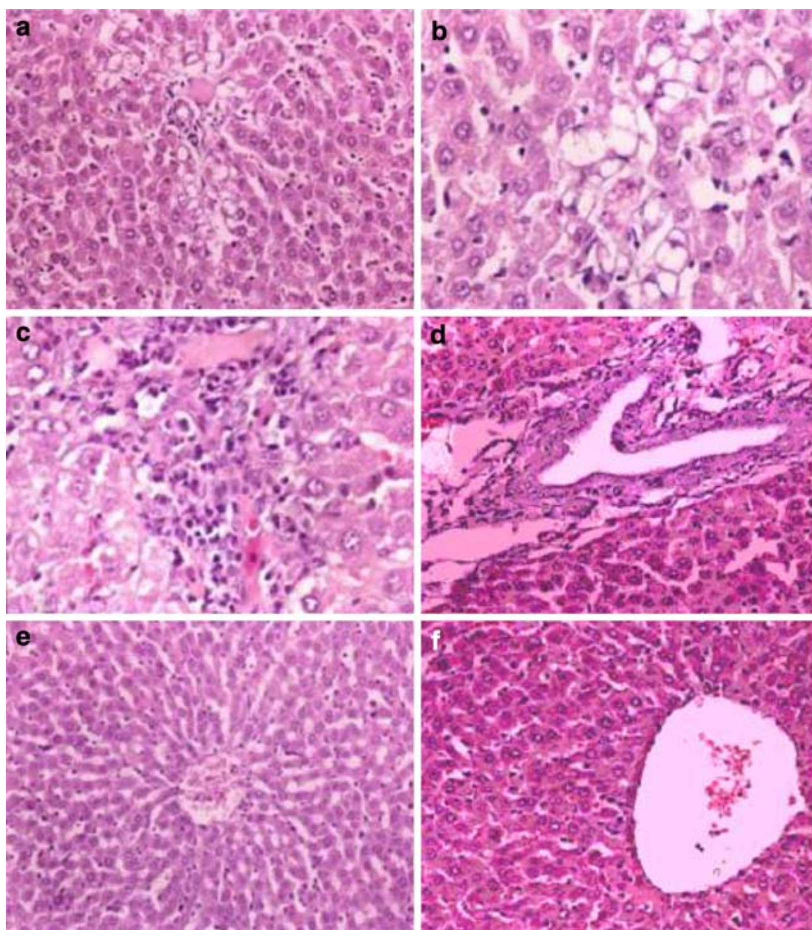

Fig. 3 Photomicrographs of liver of male rats: **a** treated with EDTA showing kupffer cell activation, sporadic cell necrosis, dilated hepatic sinusoids as well as massive vacuolations of periportal hepatocytes (H & E ×200) **b** treated with EDTA showing massive vacuolations of hepatocytes (H & E ×400). **c** Treated with EDTA showing focal area of hepatocytic necrosis associated with mononuclear leucocytic cells infiltration (H & E ×400). **d** Treated with EDTA combined with garlic showing hyperplasia of epithelial lining bile duct associated with oedema in the portal tracts. **e** and **f** treated with EDTA combined with either (ginseng alone)/or with ginseng plus garlic, respectively, showing proliferation of kupffer cells (H & E ×200)

Testis of EDTA treated rat suffered from degeneration of spermatogoneal and sertoli cells accompanied with intratubular accumulation of cellular debris (Fig. 5a). Severe alterations were observed in testis sections of rat treated with either garlic alone or EDTA plus garlic. Marked testicular degeneration with complete loss of germinal cells lining some seminiferous tubules was recorded in the examined sections (Fig. 5b). Apparent normal testis was noticed in the sections of rats treated with *P. ginseng* extract alone, EDTA combined with *P. ginseng* or EDTA combined with garlic plus *P. ginseng* extract (Fig. 5c, d).

Discussion

In 1974, the Joint FAO/WHO Expert Committee on Food Additives (JECFA 1974) recommended that CaNa2 EDTA or Na2 EDTA be permitted as food additives at doses up to an ADI of 2.5 mg EDTA/kg b.w/day, based on the facts that these additives are poorly absorbed from the gut, appear to be metabolically inert, and have a history of use in treating metal poisoning in humans. Subsequently, the International Nutritional Anemia Consultative Group (INACG 1993) and JECFA (1993b, c) evaluated the benefits and potential health concerns associated with the use of iron EDTA as an iron fortification in food.

In the current study, we evaluated the ability of *P. ginseng* extract contain 36 μg ginsenoside Rg3/mg and garlic to protect the experimental animals against the biochemical, genotoxic, and histopathological changes of EDTA. The selective dose of garlic and *P. ginseng* extract were based on our previous work (Abdel-Wahhab and Aly 2003; Mannaa et al. 2006). The significant decrease in ALT and AST reported herein in the EDTA alone-treated group indicated a marked destruction of hepatocytes (Wang et al. 2004) and the decreased level of LDH indicates the hepatocytes injury (Wang et al. 2002) as a severe effect of EDTA. Albumin level in EDTA-treated rats decreased significantly suggesting the liver cells injury. Although albumin is not a frequently used as an index to hepatic failure, its change with a steady pace giving concrete confirmation to the status of hepatic anabolism (Gao et al. 2002). Moreover, the decreased level of BUN in this group indicates protein catabolism and/or kidney dysfunction (Abdel-Wahhab et al. 2002). The significant increase in MDA accompanied with the significant decrease in SOD and GPX reported in the current study in the EDTA-treated group indicated that EDTA induced oxidative stress. These effects may be

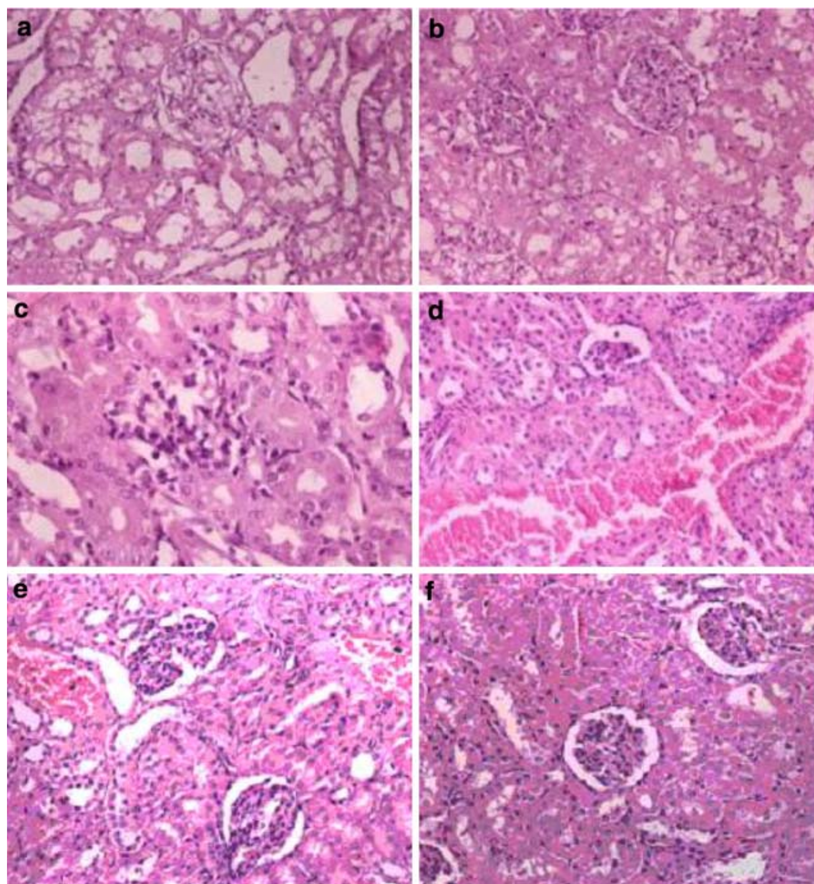

Fig. 4 Photomicrographs of kidney of male rats: **a** and **b** treated with EDTA showing marked vacuolar degeneration of epithelial lining proximal convoluted renal tubules as well as endothelial lining the glomerular tufts associated with hypertrophy of the tufts with narrowing of Bowman's spaces (H & E ×200). **c** Treated with EDTA showing small focal area of tubular necrosis replaced by mononuclear leucocytic cells (H & E ×400). **d** and **e** treated with EDTA combined with garlic or ginseng, respectively, showing congestion of renal blood vessels. **f** Treated with EDTA combined with garlic plus ginseng showing apparent normal histological structure (H & E ×200)

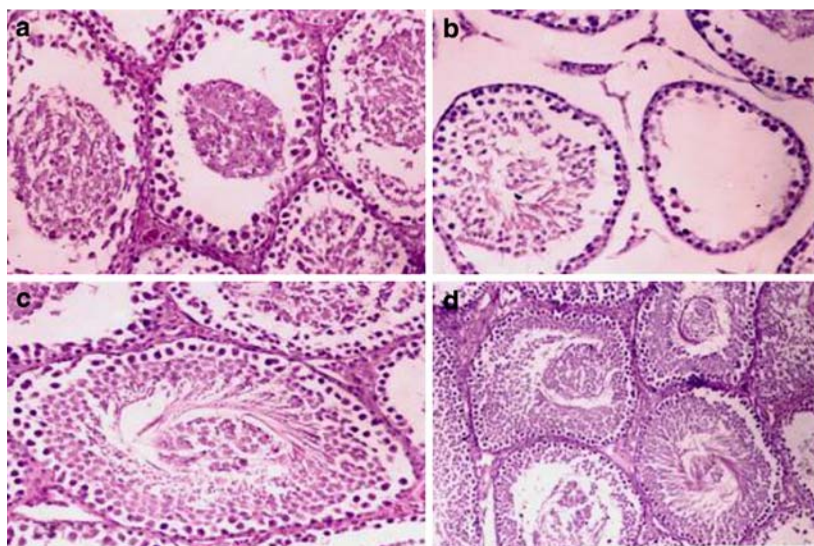

Fig. 5 Photomicrographs of testis of male rats: **a** treated with EDTA showing degeneration of spermatogoneal and sertoli cells accompanied with intratubular accumulation of cellular debris (H & E ×200). **b** Treated with EDTA combined with garlic showing marked testicular degeneration with complete loss of germinal cells lining some seminiferous tubules (H & E ×200). **c** and **d** treated with EDTA combined with ginseng and EDTA combined with garlic plus ginseng showing apparent normal testis (H & E ×200 and 100, respectively)

indirect response to EDTA as a chelating agent; consequently, the sequestering of metal ions by EDTA may be responsible for the oxidative stress (Hagiwara et al. 2006).

The clastogenicity effects of EDTA in animals were documented previously (Heindorff et al. 1983; Muralidhara and Narasimhamurty 1991). Previous study showed that EDTA was able to influence the mutagen-induced micronucleus yield (Muralidhara and Narasimhamurty 1991). The bone marrow micronucleus assay is a very sensitive and reliable test being used as a screening test for in vivo chromosomal damage (Ashby et al. 1988). In the current study, we have applied the random amplified polymorphism DNA-PCR (RAPD-PCR) to evaluate the genotoxic effects of EDTA and the possible protective effects of *P. ginseng* extract and/or garlic. Our results clearly indicated that the in vivo mutagenic activity of EDTA resulted in an increase in the

incidence of micronucleated PCE. These results confirm the earlier observations of other investigators (Manna and Das 1976; Muralidhara and Narasimhamurty 1991), who reported a higher incidence of chromosome aberrations in bone marrow cells of mice treated with EDTA and Syrian hamster embryo cells (Hagiwara et al. 2006).

Furthermore, DNA fingerprinting assay indicated the mutagenic effect of EDTA since RAPD primers displayed some changes in polymorphism band patterns of DNA including lose of stable bands in EDTA alone-treated group. According to Heindorff et al. (1983), the mechanism by which EDTA causes these genotoxicity remains poorly understood. In despite of this fact, the clastogenicity effect of EDTA may be attributed to two reasons: (1) the inhibition effect of EDTA on DNA synthesis in mammalian cells, which may be due to impairment of several enzymes involved in DNA replication; and (2) the mutagenic effect of EDTA may be attributed to the viewpoint that EDTA-induced cation deficiencies, which is responsible for the changes of chromosome morphology or mitotic abnormalities. Furthermore, FDA scientists (Lerner et al. 1986) concluded that the observed events were probably spurious indications of genotoxic potential caused by the chelation of cations that are important as enzymatic cofactors involved in DNA synthesis in the cell. In the same respect, Taylor and Jones (1972) reported that transient inhibition of DNA synthesis was observed in rat kidneys following intraperitoneal injection of various chelated forms of EDTA like calcium, sodium or manganese, but not by zinc salts of EDTA. These authors speculated that the lack of effects by the zinc EDTA salt provided evidence that zinc is required for the initiation or continuation of DNA synthesis and that the other EDTA salts probably caused a depletion of the required zinc ions in the kidney tissues.

The present study indicated that there were prominent histopathological alterations in the liver, kidney and testis of rats treated with EDTA alone. In this respect, Reuber and Schmieller (1962) claimed that the liver of rats treated with 500 mg of EDTA was normal. Furthermore, M. J. Appel (1999, submitted data) reported that histopathological examination of sections of the liver did not reveal any dose or treatment-related changes. Such observations were considered to be unremarkable and occurred either sporadically or with a uniform distribution across all groups. Reuber and Schmieller (1962) reported that the kidney showed mild to moderate renal hydropic changes with focal subcapsular swelling and proliferation in glomerular loops, which is supportive to our results. Grass et al. (1985) and Grass and Robinson (1988) stated that EDTA is a toxic agent and capable of penetrating biological membranes and tissue stroma. It might postulate that the cytotoxicity of EDTA is related to its ability to bind to intracellular metal ligands, thereby resulting in the formation of a more active toxic-inducing form (Ogundele 1998).

The natural dietary supplements have recently been demonstrated to possess many medicinal properties (Siegers et al. 1999; Loo et al. 2004). In the current study, animals treated with EDTA plus garlic, *P. ginseng* or a combination of garlic plus *P. ginseng* showed a significant improvement in all biochemical parameters tested. In a previous work, we reported that garlic contains organosulfer compounds that have protective effects against the development of many diseases (Abdel-Wahhab and Aly 2003). It succeeded to restore the level of different biochemical parameters and antioxidant enzymes toward the normal levels of the controls. Reactive oxygen species (ROS) that induced cellular damage was estimated by monitoring the LPO, which is a well-known indicator of cellular damage by oxidative stress. The current results supported the antioxidant properties of organosulfur compounds in garlic and their ability to scavenge free radical-intermediates of LPO (Abdel-Wahhab et al. 2004). In addition, the increase in hepatic GPX activity, as shown in rats treated with garlic may be facilitated by the regeneration of GPX from its oxidized form (Abdel-Wahhab and Aly 2003). This postulation is consistent with the increase in hepatic GPX level in rats treated with garlic with or without EDTA challenge. Generally, the organosulfur compounds in garlic enhance the defense mechanisms against EDTA and supported the earlier findings that organosulfur compounds enhanced the protection of liver against many toxicants (Tadi et al. 1991; Siu et al. 1996; Abdel-Wahhab and Aly 2003; Abdel-Wahhab et al. 2004).

Moreover, Panwar et al. (2005) reported that more than 95% of protopanaxadiol ginsenosides in natural *P. ginseng* are converted into ginsenoside Rg3 and Rg5; of course other protopanaxatriol ginsenosides are also converted into their congeners. This conversion makes *P. ginseng* has eight times more potent antioxidant action, 97 time potent vasodilatation action than conventional white *ginseng* and red *ginseng*. In this regard, Kim et al. (1997) reported that *ginseng* has a potent protective action against CCL_4-induced toxicity and it showed inhibitory effect on cytochrome P_{450}- associated monoxygenase activities. Therefore, it is suggested that the protective effect of *p. ginseng* is attributable to its free radical scavenging activity (Abdel-Wahhab and Ahmed 2004; Mannaa et al. 2006). Generally, these findings indicated that both garlic and *P. ginseng* extract have protective effects against EDTA-induced liver injury and they play a role in increasing the antioxidant status as well as lowering the oxidative damage of nucleic acids in the body (Abdel-Wahhab and Aly 2003; Mannaa et al. 2006).

To date, few studies were investigated the antimutagenic effects of *ginseng* against mutagenicity actions including micronucleus formation in animal models (Panwar et al. 2005; Ivanova et al. 2006). The chemoprotective action and

antimutagenic effect of the standardized *P. ginseng* extract, EFLA 400 was studied in Swiss albino mice (Panwar et al. 2005) and in C57BL/6 male mice (Ivanova et al. 2006). Lee et al. (1998) suggested that supplementation with antioxidants such as *ginseng* might protect smokers from oxidative DNA damages and could reduce cancer risk or other diseases caused by free radicals associated with smoking. In the present study, we have found that *P. ginseng* alone or combined with garlic was able to prevent the mutagenic effect of EDTA. It reduced significantly the number of MnPCEs in the bone marrow cells, as well as absence of changes in the polymorphism band patterns of the DNA induced by EDTA. Consistent with our observation, Panwar et al. (2005) reported that *P. ginseng* extract had an antimutagenic activity in mice in a dose dependent manner. On the other hand, Abraham and Kesavan (1984) reported that micronucleus formation in albino mice treated with garlic was not significantly different from control values. Consistent with these observations, we have found that rats treated with garlic showed micronucleus values similar to the control values. Kumaraguruparan et al. (2005) reported that the protective effect of garlic against *N*-methyl-NV-nitro-*N*-nitrosoguanidine-induced genotoxicity was more effective when garlic was combined with tomato than tomato alone or garlic alone. In the present study, we have also found that garlic was more impressive to prevent the mutagenicity effect of EDTA when combined with *P. ginseng* extract than garlic alone.

The histopathological examination of liver, kidney, and testis in rats treated with EDTA combined with either *P. ginseng* alone/or with *P. ginseng* plus garlic revealed a marked improvement in the histopathological picture. The histopathological results reported herein supported the findings of Salvati et al. (1996), who stated that *ginseng* stimulates spermatogenesis in rat testes and increased spermatozoa number/ml, progressive oscillating motility, increased plasma total and free testosterone, DHT, FSH, and LH levels. It is suggested that ginsenosides may have an effect at different levels of the hypothalamus-pituitary–testis axis. *Ginseng* extracts increases biosynthesis of protein and nucleic acid (Lee et al. 1993; Kim et al. 1997). Moreover, it was reported that the non-saponin components of red *ginseng* suppressed the harmful effects of ROS (O_2, H_2O_2, and, OH_2), which exercise an important role in tissue degeneration (Kim et al. 1997). Moreover, Zhang et al. (1996) showed that hydroxyl radical formed by the Fenton reaction were completely inhibited by *ginseng* extract. This antioxidant effect of *ginseng* may be responsible for its wide pharmacological actions in clinical practice by a free radical reaction-inhibition mechanism. Therefore, the protective effects of *P. ginseng* or garlic may be related to their antioxidant properties. Furthermore, earlier studies demonstrated that garlic protects against the genotoxic effects of carcinogens by modulating LPO and enhancing GSH-dependent antioxidants (Arivazhagan et al. 2000, 2001; Chandra Mohan et al. 2003; Abdel-Wahhab and Aly 2003; Abdel-Wahhab et al. 2002, 2004). The possible reason for the apparent synergistic effects of *P. ginseng* and garlic combination may be due to the presence of several phytochemicals which were reported to display both complementary and overlapping mechanism of actions, including induction of detoxification enzymes and antioxidants (Kik et al. 2001; Weisburger 2002).

In conclusion, although FDA stated that EDTA may be regarded as GRAS for the intended food uses, the current study demonstrated that EDTA (20 mg/kg b.w) induced severe biochemical, histopathological, and genotoxic changes in rats. These effects may be due to the higher dose of EDTA used in the current study, eightfold of the estimated daily intake (EDI) recommended by JECFA (1974). Both garlic and *P. ginseng* extract contain 36 μg ginsenoside Rg3/mg extract have protective effects against EDTA-induced alterations. *P. ginseng* extract was effective than garlic, whereas the combined treatment was more effective than the single treatment.

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