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Toxicological Evaluation of Fucoidan from *Undaria pinnatifida In Vitro* and *In Vivo*

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The potential toxicity of fucoidan from *Undaria pinnatifida* was investigated *in vitro* and *in vivo*. By the Ames test, fucoidan showed no mutagenicity up to 500 μL/plate, and inhibited the mutagenicity induced by 4-nitroquinoline-1-oxide, by up to 71%, compared with controls. In the bone marrow micronucleus test, fucoidan, at all levels tested, did not change the micronucleated polychromatic erythrocyte percentage ratio in mouse bone marrow cells. As an acute *in vivo* toxicity test, fucoidan from 0 to 2000 mg/kg body weight per day was administered orally to Sprague-Dawley rats for 28 days. No significant toxicological change was induced by fucoidan treatment up to 1000 mg/kg body weight per day in biochemical analyses, hematological analyses, necropsy and liver histopathology. The plasma ALT level was slightly, but significantly, increased in male rats at 2000 mg/kg/day. The consumption of fucoidan from *Undaria pinnatifida*, up to 1000 mg/kg body weight per day, may be safe in rodents, with no sign of toxicity after up to 28 days of daily administration. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: fucoidan; *Undaria pinnatifida*; Ames test; bone marrow micronucleus test; 28-day oral toxicity test.

INTRODUCTION

Fucoidan is a sulfated polysaccharide common in brown seaweeds such as Kylin, Kombu and Miyeok (*Undaria pinnatifida*). L-Fucose and sulfate are major components of fucoidans (Berteau *et al*., 2002), and minor components include glucuronic acid, xylose and galactose (Anastyuk *et al*., 2009). Recently, it has been reported that fucoidans possess various biological activities on human health. Anticoagulant and antiinflammatory properties are example biological activities of fucoidan from brown algae (Chand and Matsuhiro, 2008; Cumashi *et al*., 2007; Preobrazhenskaya *et al*., 1997; Kang *et al*., 2008; Ushakova *et al*., 2008; Medeiros *et al*., 2008). Additionally, antiviral (Baba *et al*., 1988; Mandal *et al*., 2007; Ponce *et al*., 2003), antitumor (Itoh *et al*., 1993; Maruyama *et al*., 2003; Maruyama and Yamamoto, 1984; Zhuang *et al*., 1995) and antimetastatic (Alekseyenko *et al*., 2007), anticoagulant (Dürig *et al*., 1997), antiangiogenic (Koyanagi *et al*., 2003) and antioxidant activities (Wang *et al*., 2008) have been reported. Additionally, fucoidans have also been reported to prevent *Helicobactor pylori* infection (Shibata *et al*., 2003) and to reduce the risk of associated gastric cancer (Maruyama *et al*., 2006). Thus, the production and application of fucoidans as therapeutic agents have become increasingly important topics of research.

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Nevertheless, investigations of the toxicity of fucoidans from various sources are limited; to date, two reports are available. Li *et al*. evaluated the toxicity of fucoidan extracted from *Laminaria japonica* in Wistar rats. According to their studies, animals receiving 300 mg/kg body weight per day of fucoidans from *L. japonica* showed no sign of toxicity (Li *et al*., 2005), at but 900 and 2500 mg/kg body weight per day, the blood clotting time was significantly prolonged. Additionally, an investigation of fucoidan extracted from Okinawa mozuku, a brown alga (*Cladosiphon okamuranus*), showed no significant adverse change at a dose of 600 mg/kg body weight/day of fucoidans in rats (Gideon and Rengasamy, 2008).

This study further investigated the potential toxicity of fucoidan extracted from *Undaria pinnatifida*, a very common brown algae seafood in Northeastern Asia and the most popular one in Korea. To test genotoxicity, an *in vitro Salmonella* Ames test and an *in vivo* micronucleus test with ICR mice were performed (Ames *et al*., 1975; von Ledebur and Schmid, 1973). Additionally, 28-day oral repeated administration tests in rats were performed to examine the potential harmful effects of short-term administration.

MATERIALS AND METHODS

Fucoidan. Fucoidan from *Undaria pinnatifida* was extracted as described previously, with some modifications. In general, fucoidan was isolated from *Undaria pinnatifida* by hydrolysing it in 0.05 M or 0.5 M HCl at

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80°C for 30 min and then neutralizing it with 1 M NaOH. After desalting by gel filtration, the hydrolysate was lyophilized. The chemical composition of the prepared fucoidan was 27% uronic acid, 53% monosaccharides and 7.4% sulfate. Major monosaccharides included 54% fucose and 35% galactose and the minor monosaccharides were 3% rhamnose, 4% arabinose and 1% xylose, glucose, xylose and mannose. Fucoidan was dissolved in distilled water for the experiments.

Salmonella mutagenicity test (Ames test). The Ames test was performed as described by Ames with *Salmonella typhimurium* strains TA100 and TA98 (Ames *et al*., 1975; Verschaeve and Van Staden, 2008). Test strains were provided by Professor S. S. Ham, Kangwon National University. *S. typhimurium* TA100 was used for detection of base pair mutations, and *S. typhimurium* TA98 was used for frame shift mutations. Samples of 50 μL of fucoidan (0, 125, 250, 375, 500 μg/plate, determined in a preliminary experiment) were added to a test tube containing 100 μL of bacterial culture media (OD_{600} 1.0), then phosphate-buffered saline buffer (PBS) was added up to 700 μL. The test tube was pre-incubated in a shaking incubator for 20 min at 37°C, 120 rpm. After incubation, 4 mL of top agar (45°C) containing 0.5 mM histidine/biotin was added, and then all components were spread evenly on minimal glucose agar plates. After the top agar solidified, the plates were inverted and incubated for 48 h at 37°C. His^+ revertant colonies were counted to assess the mutagenic properties of fucoidan (Merski *et al*., 2008).

Salmonella antimutagenicity test (Ames test). 4-Nitroquinoline-1-oxide (4NQO, 0.15 μg/plate, Sigma) is a carcinogen commonly as the mutagen in the Ames tests (Wang *et al*., 2007). His^+ revertant colonies were induced by 4NQO in both TA 98 and TA 100. Fucoidan (50 μL of 0, 125, 250, 375, 500 μg/plate) and 50 μL of 4NQO (0.15 μg/plate) were added to a test tube containing 100 μL of bacterial culture media (OD_{600} 1.0); then, PBS was added up to 700 μL. The His^+ revertant colonies were counted to assess the antimutagenic effects of fucoidan. The antimutagenic effects were shown by the inhibition rate (%) of fucoidan against 4NQO according to the equation:

$$\text{Inhibition rate (\%)} = (M - S_1)/(M - S_0) \times 100$$

where M is the number of revertant colonies on the positive control plates with the mutagen, S_1 is the number or revertant colonies on the sample plates with the mutagen and S_0 is the number of revertant colonies on the negative control plates (spontaneous).

Bone marrow micronucleus assay. The bone marrow slides were prepared as described by Holden *et al.* (1997) with some modifications. Fucoidan was administered orally (0, 250, 500, 1000, 2000 mg/kg) to 5 week-old ICR male mice (Samtako, Korea). Mitomycin C was administered (2 mg/kg) as a positive control. After 36 h, the animals were killed. The bone marrow cells from the femur were obtained using a 22G needle fitted to a syringe and then homogenized with fetal bovine serum (FBS; Welgene Co, Korea). The suspension was centrifuged and the pellet was suspended in the residual FBS and a drop of it was smeared onto a clean, grease-free slide and fixed in absolute methanol for 5 min. The slides were stained with 5% Giemsa in Sorensen's phosphate buffer (pH 6.8). The cytotoxicity of fucoidan was determined from the micronucleated polychromatic erythrocyte percentage ratio (MNPCEs) per polychromatic erythrocytes (PCEs). In total, 500 polychromatic erythrocytes and/or normochromatic erythrocytes (NCEs) were scored for %PCEs/(PCEs + NCEs).

28-day oral repeated administration test. Six-week-old Sprague-Dawley (SD) male and female rats (Samtako, Korea) were grouped randomly and acclimated for a week before the experiment. The animals were housed separately in 22.5 W × 20 L × 19 H cm stainless cages in an air-conditioned room at 20 ± 3°C, relative humidity 50 ± 10%, with a 12/12 h light/dark cycle. During the experiment, the animals were allowed access to a NIT31 rodent diet (Samtako, Korea) and water. Animals were killed after 16 h of fasting.

The animal protocol was designed according to the guidelines of the National Institute of Toxicological Research, Korea. Animal care and handing were performed under protocols approved by the Committee on Animal Experimentation and Ethics of Korea University (Sung *et al*., 2009).

Oral administration of fucoidan. In female and male groups, five rats were assigned to each fucoidan dosage group (0 [vehicle], 250, 500, 1000, 2000 mg/kg/day). During the 28-day feeding period, fucoidan was administered orally with a zonde once every morning at the same time each day. The health condition, clinical symptoms and mortality of the animals were checked twice daily, before and after the administration, as described previously (Okazaki *et al*., 2002). Body weights, food consumption and water consumption were recorded once a week. The animals were killed by avertin injection (2,2,2 tribromoethanol; Sigma, St Louis, MO, USA) after 16 h of fasting.

Biochemical and hematological parameters. Blood samples were taken with cardiac puncture for biochemical and hematological analysis. Plasma samples were obtained by centrifugation (15000 rpm, 10 min, 4°C). The biochemical parameters determined included total, low-density-lipoprotein (LDL) and high-density-lipoprotein (HDL) cholesterols, alanine transaminase (ALT), aspartate transaminase (AST), glucose and triglyceride concentrations, and were measured with a Cobas C111 analyser (Roche, IN, USA), an automatic clinical chemistry analyser. Hematological parameters included hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution with (RDW), white blood cell count (WBC), red blood cell count (RBC), and were measured at the Blood Chemistry Laboratory at Korea University Anam Hospital (Seoul, Korea).

Necropsy. At necropsy, the animals were weighed and then killed. Brain, heart, thyroid, liver, lung, kidney, adrenal, spleen, testis and ovary were taken and weighed. Detailed histopathological and morphological observation was performed by a pathologist at the Korea University Anam Hospital. Liver tissues were fixed with 10% (v/v) formaldehyde, sectioned and stained with

hematoxylin and eosin (H&E), and images were obtained at 200× and 400× magnification.

Statistical analyses. Statistical comparisons between the sample group and the control group were conducted using Student's *t*-test. The results are expressed as the mean ± SEM unless indicated otherwise. Values of $p < 0.05$ were deemed to be statistically significant.

RESULTS

Salmonella mutagenicity and antimutagenicity test

Fucoidan treatment did not induce significant dose-dependent revertant colonies at any of the doses tested, compared with the spontaneous revertant colonies formed in the control group, in either *S. typhimurium* TA 98 or TA 100 strains (Table 1). Additionally, fucoidan treatments exhibited dose-dependent antimutagenic effects in both TA98 and TA100 against 4NQO as a mutagen. The maximum inhibition of mutagenicity ratio with fucoidan treatment was 76% (Fig. 1).

Micronucleus test with ICR mice

No significant clinical symptom was observed during the experiment, and no mouse died. Fucoidan administration at the doses tested caused no significant change in %MNPCE or %PCE (PCE + NCE), compared with the negative control groups, while mitomycin C administration, the positive control, caused significant changes in %MNPCE and %PCE (PCE + NCE), as expected (Table 2).

Table 1. Mutagenicity of fucoidan in *Samonella typhimurium* TA98 and TA100

	Fucoidan His+ revertants/plate	
Dose (μL/plate)	TA98	TA100
0	22 ± 2	174 ± 4
125	19 ± 2	178 ± 12
250	23 ± 3	162 ± 27
375	24 ± 2	161 ± 7
500	21 ± 2	177 ± 30

Each value represents the mean ± SE of three plates.

28-day oral repeated administration test

Clinical symptoms. No specific clinical symptom was observed in any animal during the 28 days. Body weight, water and food consumption were observed for 4 weeks and recorded once a week (data not shown). There was no significant difference or trend in body weight, or water or food consumption among the groups.

Hematological and biochemical parameters. Fucoidan administration, at up to 1000 mg/kg/day, caused no significant difference in hematological or biochemical parameters (data not shown). However, the ALT and triglyceride levels in the 2000 mg/kg/day male groups were significantly higher (34.0 ± 0.7 and 56.8 ± 4.7, $p < 0.05$), and the total and HDL cholesterol levels in the 2000 mg/kg/day female groups were significantly higher (56.8 ± 4.7, $p < 0.05$). Additionally, MCHC in the 2000 mg/kg/day female groups was significantly lower (34.0 ± 0.0).

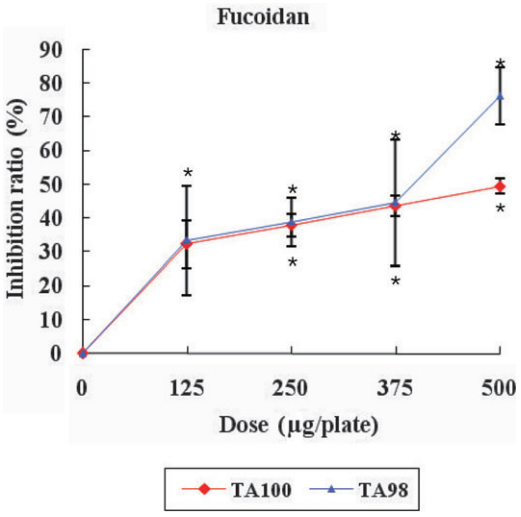

Figure 1. Antimutagenicity of fucoidan in *Salmonella typhimurium* T98 and TA100. * $p < 0.05$, versus the negative control.

Table 2. The bone marrow micronucleus test in male ICR mice administered fucoidan

				Mean ± SE	
Compound	Dose (mg/kg)	No. of animals	Exposure time (h)	% MNPCE	%PCE (PCE + NCE)
Fucoidan	2000	5	36	0.14 ± 0.02	60.48 ± 6.00
Fucoidan	1000	5	36	0.14 ± 0.02	63.20 ± 1.80
Fucoidan	500	5	36	0.14 ± 0.04	62.24 ± 1.50
Fucoidan	250	5	36	0.12 ± 0.02	64.96 ± 1.33
Negative control	0	5	36	0.14 ± 0.02	59.56 ± 7.34
Mitomycin C	2	5	36	3.80 ± 0.57[a]	33.47 ± 7.66[a]

PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes; %MNPCE, [Micronucleated polychromatic erythrocytes/polychromatic erythrocytes] × 100; %PCE (PCE + NCE), PCE = [PCE/(PCE + NCE)] × 100.

[a] $p < 0.05$ compared with negative control.

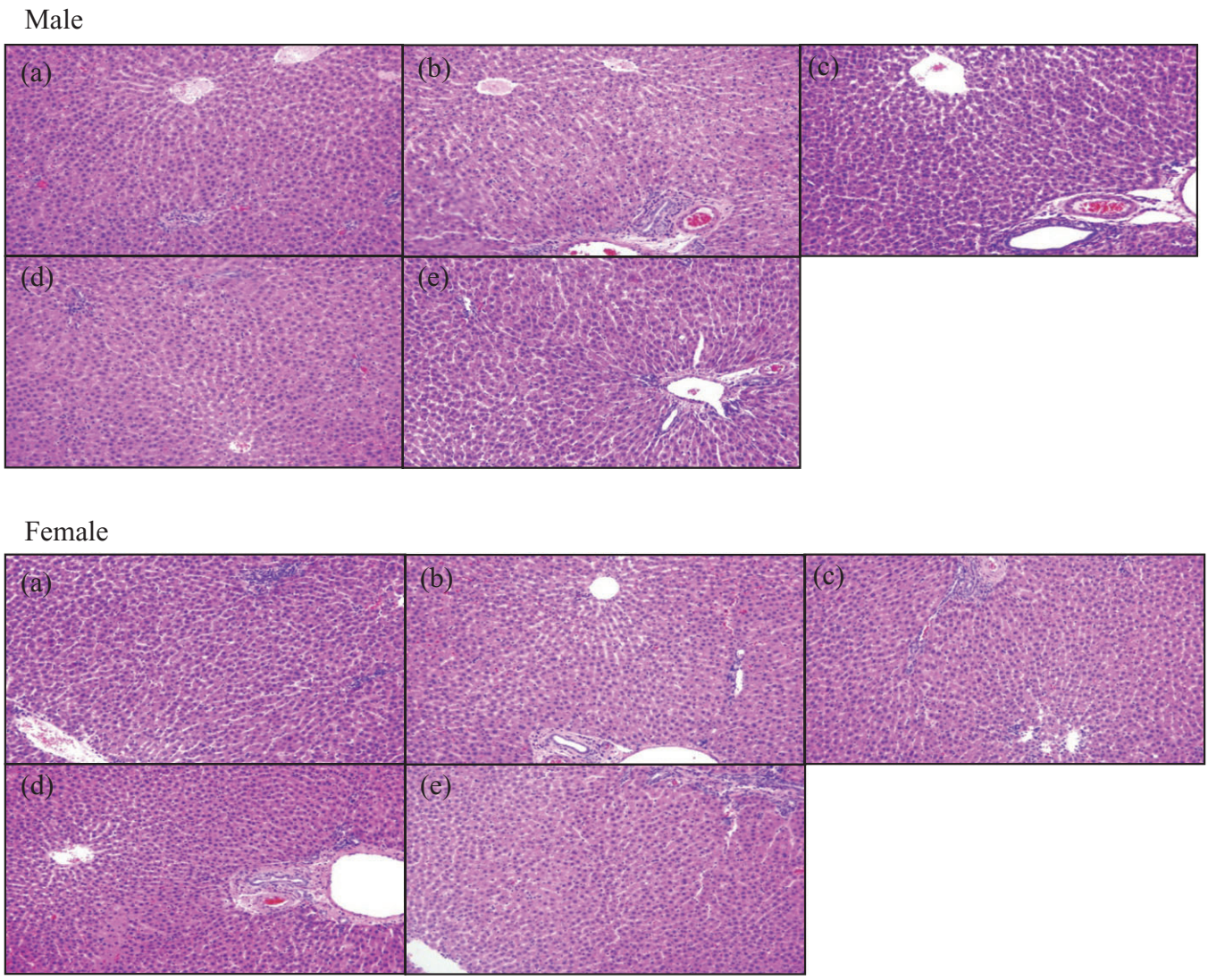

Figure 2. Representative photomicrographs of livers from male and female rats after 4 weeks of fucoidan administration (a) 2000 mg/kg, (b) 1000 mg/kg, (c) 500 mg/kg, (d) 250 mg/kg, (e) control (hematoxylin-eosin stain, 200×).

Necropsy and representative microscopic observation of the livers. Major organs, including brain, heart, thyroid, liver, lung, kidney, adrenal, spleen, testis and ovary, were sectioned for gross examination. It was observed that fucoidan administration up to 1000 mg/kg/day cause no significant difference in organ weight or pathology. However, thyroid weights at 2000 mg/kg/day were significantly altered in both sexes (data not shown). The H&E stained liver sections were observed for the histochemical analysis (200× magnification). No outstanding histopathological finding was observed in any of the fucoidan-treated groups, compared with those of the control livers (Fig. 2).

DISCUSSION

The potential toxicity of fucoidan from *Undaria pinnatifida* was investigated. Potential *in vitro* mutagenic activities were assessed using Ames tests, which were performed with *S. typhimurium* TA 98 and TA 100 strains on agar plates containing 0500 μg/mL fucoidan. In the mutagenicity tests, there was no dose-dependent increase in the number of revertant colonies in groups treated with fucoidan, compared with the negative controls. Fucoidan treatment even showed dose-dependent antimutagenic activity against a mutagen, 4-NQO (0.15 μg/plates), up to 71%, compared with the control TA 98 strain. These findings suggest that fucoidan extracted from *Undaria pinnatifida* did not cause mutagenicity and may even have beneficial antimutagenic activity. These protective effects may support the known anticancer activity of fucoidans.

In the micronucleus test, we examined whether fucoidan caused DNA damage in bone marrow cells in ICR male mice *in vivo*. Mitomycin C, the positive control, caused structural damage to bone marrow cells, increased %MNPCE and reduced %PCE (PCE + NCE) values. However, none of the tested doses of fucoidan extracted from *Undaria pinnatifida* significantly altered the %MNPCE and %PCE (PCE + NCE) values, compared with the negative controls. These results suggest that the oral administration of fucoidan from *Undaria pinnatifida* did not disrupt the normal formation of erythrocytes, did not damage chromosomal structure or

chromosome numbers, and that the intake of less than 2000 mg/kg body weight per 36 h in rodents did not cause genotoxicity and was safe.

As an oral acute toxicity test, a 28-day oral repeated administration test was performed in SD rats. Fucoidan was administered by oral gavage every day at the same hour. In both female and male groups, no significant change was observed in body weight, or food or water consumptions during the 28 days of feeding. In groups receiving less than 1000 mg/kg of body weight/day fucoidan, there were no significant changes in hematological or biochemical parameters. However, the administration of more than 2000 mg/kg of body weight/day fucoidan induced significant changes in ALT, triglycerides, cholesterol and HDL. Also, in groups treated with 2000 mg/kg body weight/day fucoidan, MCHC changed significantly in female animals.

At necropsy, the relative and absolute weights were not different in groups receiving less than 1000 mg/kg body weight/day fucoidan, and there were no visible change in gross lesions. However, the relative weights of the thyroid were increased significantly with 2000 mg/kg body weight/day fucoidan, in both sexes. For a more specific observation, microscopic analysis was performed in liver sections. No difference was observed in hepatocyte morphology, such as vacuolation, occasional double nuclei or swollen hepatocytes.

There have been two previous *in vivo* toxicological investigations of fucoidan. Li *et al*. (2005) studied fucoidan extracted from *Laminaria japonica* in Wistar rats for 6 months and reported no significant toxicological change with 300 mg/kg body weight per day fucoidan administration, though prolonged clotting times were seen at 900 and 1500 mg/kg body weight/day administration. They concluded that fucoidan from *Laminaria japonica* had no adverse effect at 300 mg/kg body weight/day. In another study, Gideon and Rengasamy (2008) investigated the toxicity of fucoidan from *Cladosiphon okamuranus* in rats after oral administration for 3 months. They found no significant change induced by fucoidan, up to 600 mg/kg body weight per day. Although the experimental designs and settings differed slightly between the studies, these results, including ours, suggest that the potential toxicity of fucoidan from various sources could be different and may require individual confirmation to optimize biological efficacy, while minimizing any potential harmful effects.

Although the liver morphology was normal, the results did reveal alterations in the liver enzymes, ALT levels and lipoprotein metabolism, including plasma triglyceride and cholesterol levels. These suggest potential hepatotoxicity of fucoidan from *Undaria pinnatifida*. This should be examined in future studies.

In conclusion, fucoidan at 500 μL/plate displayed no mutagenicity and did show antimutagenic activity against 4-nitro-quinoline-1-oxide *in vitro*. Moreover, *in vivo*, fucoidan caused no structural or numerical damage to chromosomes. Through 28 days of oral repeated administration to SD rats, it was found that less than 1000 mg/kg body weight/day of fucoidan caused no toxicological sign in biochemical analyses, hematological analyses, necropsy or liver histopathology. Fucoidan from *Undaria pinnatifida* did not cause genotoxicity or acute toxicity at a dose of up to 1000 mg/kg body weight/day; thus, it is safe for use in rodents.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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