Regulatory Toxicology and Pharmacology 61 (2011) 373–380

Contents lists available at SciVerse ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Evaluation of the genotoxic potential of standardized extract of *Glycyrrhiza glabra* (GutGard™)

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ARTICLE INFO

Article history:
Received 7 June 2011
Available online 13 October 2011

Keywords:
Glycyrrhiza glabra
Bacterial reverse mutation test
Safety
Chromosome aberration test
Micronucleus test

ABSTRACT

Glycyrrhiza glabra Linn. (licorice) is widespread throughout the Mediterranean region and certain areas of Asia. Historically, the dried rhizome and root of the plant were used by the Chinese, Egyptian, Greek, Indian, and Roman civilizations as expectorant and carminative. In the modern medicinal system, licorice is used to treat liver ailments, dyspepsia, bronchitis, rheumatoid arthritis etc. Despite the extensive pharmacological applications, the genotoxic potential of *G. glabra* extract (GutGard™) has not been evaluated. Hence, this study was conducted to investigate the genotoxic potential of GutGard™ using battery of *in vitro* test systems: bacterial reverse mutation test (Ames II™), chromosome aberration (CA) and micronucleus (MN) tests. GutGard™ did not show significant increase in number of revertant colonies in *Salmonella typhimurium* strains (TA98 and TAMix) with/without S9 fraction. In CA and MN studies, GutGard™ did not show clastogenic effect at 4 and 18 h treatments with and without S9 fraction. Results indicated that GutGard™ is not mutagenic in a battery of genotoxicity tests.

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1. Introduction

Glycyrrhiza glabra (*G. glabra* L.; Family: Papilionaceae/Fabaceae) is a traditional medicinal herb, which grows in various parts of the world (Hayashi et al., 1990). The roots and rhizomes of *G. glabra* have been widely used in medicines for its unique and diverse pharmacological properties viz., antiviral, anticancer, anti-ulcer, anti-diabetic, anti-inflammatory, anti-oxidant, immuno-stimulant, anti-allergenic etc. (Zore et al., 2008; Dong et al., 2007). In addition, the root is also used as flavoring and sweetening agents for tobaccos, chewing gums, candies, toothpaste and beverages (Asada et al., 2000).

Phytochemical investigations have demonstrated that the major bioactive components of *G. glabra* root are flavonoids and pentacyclic triterpene saponin, including liquiritin, liquiritigenin, isoliquiritigenin, liquiritin apioside, glycyrrhizin and glycyrrhizic acid (Kamei et al., 2003). Among these phytoconstituents, glabridin [(R)-4-(3,4-dihydro-8,8-dimethyl)-2H,8H-benzo[1,2-b:3,4-b′]dinyran-3yl]-1,3-benzenediol] is the major active flavonoid present in the extract. Wide range of biological activities, including antimicrobial, anti-inflammatory and cardiovascular protective activities of glabridin have been reported previously (Fuhrman et al., 1997; Fukai et al., 2003; Zhou et al., 2004; Kang et al., 2005).

GutGard™ is a standardized de-glycyrrhizinated extract of *G. glabra* root. In our previous works, we reported the anti-ulcer and anti-inflammatory properties of GutGard™ (Mukherjee et al., 2010; Chandrasekaran et al., 2011; Thiyagarajan et al., 2011). In a recent randomized, double-blind, placebo-controlled clinical study in human, GutGard™ was reported to alleviate the symptoms of functional dyspepsia (Raveendra et al., 2011). Our literature survey revealed that, there is little information about general toxicity, acute, chronic and genotoxicity of de-glycyrrhizinated and flavonoid rich *G. glabra* extract.

In this lieu, we have assessed the genotoxic potential of GutGard™, in bacterial reverse mutation test, chromosome aberration (CA) test and micronucleus (MN) test. This study was carried out in compliance with the Test Guidelines of the Organization for Economic Cooperation and Development (OECD, 1997a,b, 2004).

2. Materials and methods

2.1. Preparation of Glycyrrhiza glabra extract (GutGard™) and analysis of chemical constituents

GutGard™ extract was prepared as per our standardized method. In brief, the dried roots of *G. glabra* were extracted using acetone in 1:4 proportions at room temperature. This procedure was repeated thrice and the resultant mixture was mixed and filtered under vacuum at temperature <55 °C until a thick paste was obtained. The extract was further filtered and dried under vacuum

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doi:10.1016/j.yrtph.2011.10.002

(<65 °C, 500 mm Hg) to get the de-glycyrrhizinated extract of *G. glabra* (56 g). Thereafter, the phytoconstituents present in the extract were isolated and identified by HPLC analysis as described previously (Chandrasekaran et al., 2011).

2.2. Bacterial reverse mutation test (Ames II™ tests)

GutGard™ was tested for its possible mutagenic effect using Ames II™ tests. Ames II™ tests were performed using a fluctuation procedure in a liquid micro titer (colorimetric readout) plate and consists of the 'strains' TAMix and TA98. TAMix is a mixture of the *Salmonella typhimurium* strains viz., TA7001, TA7002, TA7003, TA7004, TA7005 and TA7006. TAMix is used for the detection of base pair substitutions and TA98 to detect frameshift mutations. A comparison of the test outcome of the different investigators resulted in an inter-laboratory consistency of 89.5%. Owing to the high concordance between the two test systems, and the low inter-laboratory variability in the Ames II™ assay results, the Ames II™ is an effective screening alternative to the standard Ames test (Maron and Ames, 1983), requiring less test material and labor (Flückiger-Isler et al., 2004; Kamber et al., 2009).

The Ames II™ kit comprises of bacterial strains (TA98 and TA-Mix), growth medium, exposure medium, ampicillin and reversion indicator medium (Xenometrix, Allschwil, Switzerland). 10 µL of freshly thawed strains were inoculated in 10 mL of growth medium in the presence of ampicillin (50 µg/mL). The cultures were grown overnight (16 h) in an orbital shaker set at 37 °C and 180 rpm.

GutGard™ was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 125 mg/mL, which was serially diluted to the indicated concentration using DMSO. Ames II™ assay was performed in the presence and absence of Aroclor™-1254 induced male Sprague–Dawley rat liver microsomal enzyme (S9) mix (Moltox, Boone, North Carolina). Positive control chemicals used in the experiment are described in Table 1.

Dose range finding study was performed with the tester strains at a concentration range of 16–5000 µg/mL (OECD, 1997a). GutGard™ showed precipitation and cell toxicity at a concentration of 5000 and 1590 µg/mL, respectively. Hence, GutGard™ was tested at 1.6, 5, 16, 50, 158, 501 µg/mL in the confirmatory mutagenicity assay. 4% DMSO was used as the vehicle control, which neither showed cytotoxicity nor impede the study. The entire experiment was performed strictly following the prescribed protocol by Flückiger-Isler et al. (2004). The number of positive (yellow) wells out of 48 wells per replicate and dose was compared with the number of spontaneous revertants obtained in the DMSO control.

Results were expressed as mean and standard deviation of revertant colonies from each triplicate of plates per concentration. The baseline for determining fold induction was calculated from the mean of spontaneous number of positive wells plus one standard deviation.

2.3. Cell culture

Chinese Hamster Ovary (CHO-K1) cells were procured from American Type Culture Collection (ATCC number CCL-61™, Rockville, MD, USA). The modal chromosome number of the CHO-K1 cells is 20 ± 2 with a doubling time of approximately 13 h. Cells were cultured in Ham's F-12 K media (GIBCO Life Technologies, NY, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, USA) and 10 mM sodium pyruvate (Sigma–Aldrich Co., MO, USA). Cells were incubated at 37 °C in 5% CO_2 atmosphere.

2.4. CC_{50} determination in CHO-K1 cells

CC_{50} can be defined as test sample (GutGard™) concentration which reduced the cell viability to 50% of the vehicle control value. To determine CC_{50} of GutGard™, CHO-K1 cells were seeded at 5×10^4 cells per well in 96 well plate and incubated for 24 h. GutGard™ was treated at the indicated concentrations with/without S9 in the specified duration of time points. After incubation, final concentration of 500 µg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added and incubated for 1 h. The formosan crystals formed by viable cells were dissolved in DMSO and read at 570 nm using Versamax spectrophotometer (Molecular devices, USA). The relative cell count was determined by comparing the cell counts in GutGard™ and vehicle control cultures. Using the above study, the dose range was designed to consider the solubility and cytotoxicity; CC_{50} for 4 h + S9 and –S9 were 40 µg/mL (Figs. 1 and 2) and 14.6 µg/mL for 18 h –S9 (Fig. 3). The highest concentrations obtained in both the studies were aimed at producing 50–60% of cytotoxicity, while the corresponding test concentrations were separated by a spacing square root of 10 (OECD, 1997b).

2.5. Treatment procedure for chromosome aberration and micronucleus tests

Chromosome aberration study and micronucleus tests were conducted according to the OECD guidelines (OECD, 1997b,

Table 1
Vehicles, positive controls and co-factor mix for genotoxicity evaluation.

Chemical	Symbol	Supplier	Solvent
Vehicle			
Dimethylsulfoxide	DMSO	Sigma–Aldrich Chemical Co. (St. Louis, MO, USA)	–
Bacterial reverse mutation test			
2-Nitrofluorene	2-NF	Sigma–Aldrich Chemical Co. (St. Louis, MO, USA)	DMSO
4-Nitroquinoline *N*-oxide	4NQNO	Sigma–Aldrich Chemical Co. (St. Louis, MO, USA)	DMSO
2-Aminoanthracene	2AA	Sigma–Aldrich Chemical Co. (St. Louis, MO, USA)	DMSO
Chromosome aberration and micronucleus test			
Ethyl Methane Sulfonate	EMS	Sigma–Aldrich Chemical Co. (St. Louis, MO, USA)	DPBS
Benzo [a] pyrene	B[a]P	Sigma–Aldrich Chemical Co. (St. Louis, MO, USA)	DMSO
Co-factor mix			
Glucose-6-phosphate	G-6-PO_4	Sigma–Aldrich Chemical Co. (St. Louis, MO, USA)	Sterile water
Di-sodium hydrogen phosphate	Na_2HPO_4	Himedia (Mumbai, India)	
Sodium di-hydrogen phosphate	NaH_2PO_4	Himedia (Mumbai, India)	
Potassium chloride	KCl	Himedia (Mumbai, India)	
Magnesium chloride	$MgCl_2$	Himedia (Mumbai, India)	
Nicotinamide Adenine Dinucleotide Phosphate	NADP	Sigma–Aldrich Chemical Co. (St. Louis, MO, USA)	
Metabolic activation			
Aroclor™-1254 induced male Sprague–Dawley rat liver microsomal enzyme	S-9	Moltox (Boone, North Carolina, USA)	Co-factor mix

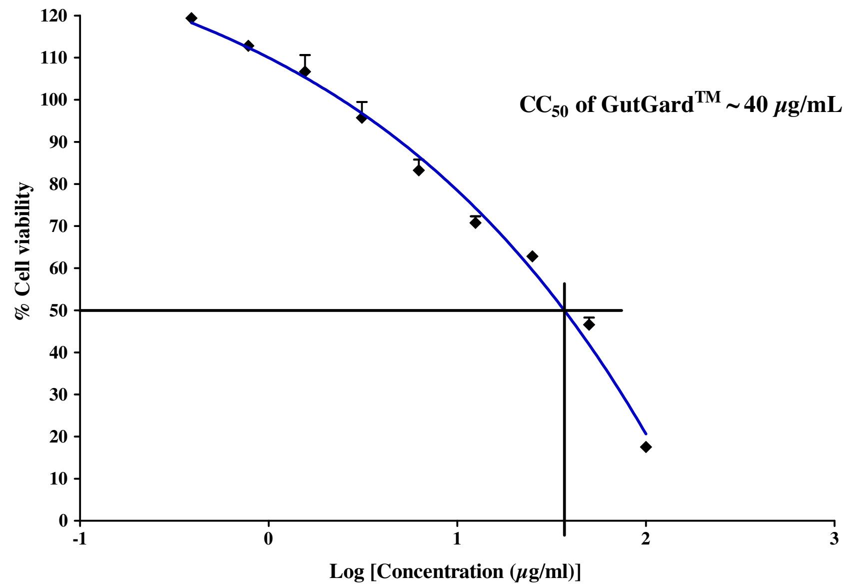

Fig. 1. Effect of GutGard™ on viability of CHO-K1 cells in short term treatment for 4 h with metabolic activation (+S9).

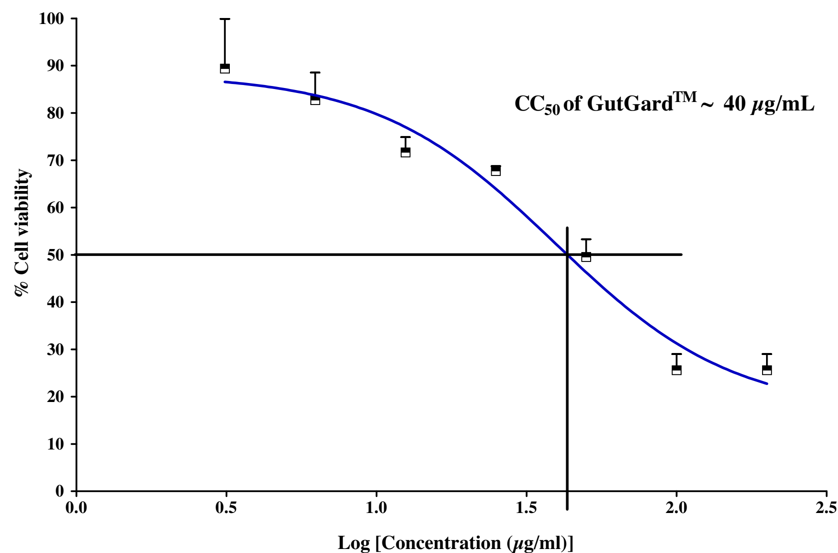

Fig. 2. Effect of GutGard™ on viability of CHO-K1 cells in short term treatment for 4 h without metabolic activation (−S9).

2004). The CHO-K1 cells were seeded at 7.5×10^5 cells/plate and incubated overnight. Post-incubation the cells were treated with GutGard™ at concentrations 4, 12.6 and 40 μg/mL for 4 h with or without S9 and 1.46, 4.6 and 14.6 μg/mL for 18 h without S9. Ethyl methanesulfonate (EMS) at 500 μg/mL (for 4 and 18 h without S9) and Benzo(a)pyrene (B[a]P) at 20 μg/mL (4 h with S9) were used as positive controls. 1% DMSO was used as the vehicle control. The cultures were run in duplicates.

2.6. Preparation of metaphase cells

Three hours prior to the harvest time, colchicine at a final concentration of 0.3 μg/mL was added to block the cells at metaphase stage. At the end of incubation, cells were washed using Dulbecco's Phosphate Buffered Saline (DPBS) and collected after trypsinization by centrifugation, treated with potassium chloride (0.075 M) followed by fixing in methanol:acetic acid (3:1,v/v) for 3–4 h. Two to three drops of fixed cell suspension from the height of 2 m were dispensed onto the surface of multi cleaned cold micro slides; air dried and stained with 5% Giemsa solution (Himedia, Mumbai) for 15 min. The slides were coded and examined under microscope (Olympus CX – 41, Japan) for a total of 200 metaphases per treatment. Structural chromosome aberrations including both chromosomes and chromatid types (i.e., break, deletion, fragments, ring, dicentric, rearrangements and exchanges) were identified and recorded as per Savage, 1975. Gaps were recorded but not included in the aberration frequency. Any metaphase with one or more aberration, regardless of its type, was classified as one aberrant metaphase. The level of cytotoxicity was determined by the reduction in mitotic index when compared to vehicle control. Mitotic index was calculated as follows (Umar-Tsafe et al., 2004): Mitotic index = (No. of dividing cells/Total no. of cells) × 100.

2.7. Preparation of cells for micronucleus test

Cytochalasin B (3 μg/mL) was added after treatment for 4 h (+S9/−S9) and simultaneously along with the test substance for 18 h (−S9) treatment procedures to arrest the cells at cytokinesis

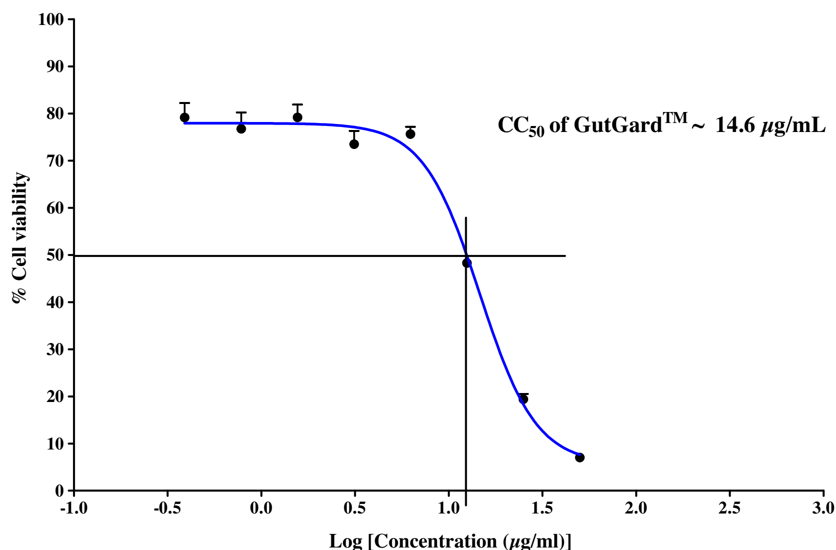

Fig. 3. Effect of GutGard™ on viability of CHO-K1 cells in long term treatment for 18 h without metabolic activation (−S9).

stage. After incubation the cells were harvested and processed for scoring using the above outlined standardized procedure except that two to three drops of fixed cell suspension were dropped to the slides from the height of 30 cm. The induction of micronucleus was determined in 2000 binucleated cells with well preserved cytoplasm. The criteria for selecting binucleated cells were based on the report by Fenech, 2000. Cytotoxicity was measured in terms of Cytokinesis Block Proliferation Index (CBPI): CBPI = No. of mononucleated cells + 2 (No. of binucleated cells) + 3 (No. of multinucleate cells)/Total number of cells. CBPI value equal to one was considered 100% cytotoxicity (Erexson et al., 2001).

2.8. Statistical Analysis

All the data are represented as mean ± SD. Statistical evaluation of the result was performed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, USA). Differences between the treated and vehicle control groups were analyzed using the one way analysis of variance (ANOVA). The result of the statistical evaluation was regarded as significant when the p value <0.05 (Erexson et al., 2001).

3. Results

3.1. Bacterial reverse mutation test

Ames II™ tests are potent assays to determine the ability of test substance to induce the reversion of histidine auxotrophy to prototrophy. The results of the mutagenicity testing are presented in Table 2 and 3. GutGard™ did not induce significant number of revertant colonies in two bacterial strains (TA98 and TAMix) both in the presence and absence of metabolic activation. Nevertheless, on treatment with the positive controls, 2-NF (2-Nitrofluorene) and 4-NQO (4-Nitroquinoline-N-oxide) significantly amplified the number of histidine revertants in the absence of metabolic activation. S9 dependent mutagen, 2-AA (2-Aminoanthracene) also displayed significant increase in the number of revertant colonies in both the *S. typhimurium* mutant strain. The number of histidine revertants in vehicle and positive controls was in accordance with the acceptable mean revertant values. Therefore, the Ames II™ tests for GutGard™ is negative.

3.2. In vitro chromosome aberration analysis in CHO-K1 cells

Possible clastogenic effect of GutGard™ was evaluated using mammalian chromosome aberration test using CHO-K1 and the results were given in the Tables 4A–4C. 4 and 18 h treatment with indicated concentration of GutGard™ both in presence and absence of S9-mix did not induce significant number of structural chromosomal aberrations. The aberration rates at all the tested concentrations of GutGard™ were within the DMSO control data. Treatment with DMSO only (vehicle control) also resulted in chromosomal aberration incidences similar to those from historical negative control data. Positive controls EMS (both 4 and 18 h exposure, −S9) and B[a]P (4 h exposure, +S9) demonstrated significant induction of structural chromosomal aberrations indicative of the validity of the experiments.

Mitotic Index is a measure for the proliferation status of a cell population. Mitotic index was analyzed on the same slides that were used for the estimation of chromosome aberrations. GutGard™ significantly decreased the mitotic index value at the highest tested concentration indicating its cytotoxicity. Overall, results indicate that GutGard™ is not clatogenic.

3.3. In vitro micronucleus analysis in CHO-K1 cells

The frequency of micronucleus formation and CBPI obtained in micronucleus test is shown in Table 5A and 5B, respectively. Results revealed that GutGard™ did not produce significant aneugenic activity after treatment with any of the tested concentrations when compared to the concurrent vehicle control cells ($p < 0.05$) both with and without metabolic activation. With respect to the cytotoxic effects of the test compound on CHO-K1 cells, as measured by CBPI, a reduction in cell proliferation has also been observed, reaching statistical significance at the highest tested concentration of GutGard™ ($p < 0.05$) compared to vehicle control. Therefore, our results indicate that GutGard™ is not aneugenic.

4. Discussion

Occupancy of herbal remedies is an increasing trend in the field of complementary and alternative medicine (CAM). The use of ethnomedical information has significantly contributed to healthcare worldwide through the isolation of bioactive compounds for direct

Table 2
Colony counts of histidine revertants in the *Salmonella typhimurium* strain: TA98.

Treatment	Concentration (μg/mL)	Number of histidine revertant colonies					
		S9	Individual colony counts			Mean ± SD	Fold induction over baseline
			1	2	3		
GutGard™	1.6	–	2	3	0	1.67 ± 1.53	–
		+	2	1	1	1.33 ± 0.58	–
	5	–	0	2	2	1.33 ± 1.15	–
		+	1	2	0	1.00 ± 1.00	–
	16	–	1	1	0	0.67 ± 0.58	–
		+	1	2	2	1.67 ± 0.58	–
	50	–	2	0	0	0.67 ± 1.15	–
		+	2	1	1	1.33 ± 0.58	–
	158	–	1	1	0	0.67 ± 0.58	–
		+	4	3	1	2.67 ± 1.53	–
	501	–	2	1	0	1.00 ± 1.00	–
		+	2	3	0	1.67 ± 1.53	–
Vehicle control	DMSO (4%)	–	1	3	2	2.00 ± 1.00	–
		+	2	3	2	2.33 ± 0.58	–
Positive control (2NF + 4NQNO)	2NF (2) + 4NQNO (0.5)	–	48	48	48	48.00 ± 0.00**	16.00
Positive control (2AA)	2AA (5)	+	48	48	48	48.00 ± 0.00**	16.49

2NF, 2-Nitrofluorene; 4NQNO, 4-nitroquinoline-*N*-oxide; 2AA, 2-aminoanthracene.
** $p < 0.05$.

Table 3
Colony counts of histidine revertants in the *Salmonella typhimurium* strain: TAMix.

Treatment	Concentration (μg/mL)	Number of revertants colonies					
		S-9	Individual colony counts			Mean ± S.D	Fold induction over baseline
			1	2	3		
GutGard™	1.6	–	1	1	1	1.00 ± 0.00	–
		+	3	0	3	2.00 ± 1.73	–
	5	–	1	1	3	1.67 ± 1.15	–
		+	1	1	2	1.33 ± 0.58	–
	16	–	1	0	0	0.33 ± 0.58	–
		+	5	2	1	2.67 ± 2.08	–
	50	–	1	0	1	0.67 ± 0.58	–
		+	1	1	1	1.00 ± 0.00	–
	158	–	0	1	0	0.33 ± 0.58	–
		+	0	1	1	0.67 ± 0.58	–
	501	–	1	0	0	0.33 ± 0.58	–
		+	1	1	0	0.67 ± 0.58	–
Vehicle control	DMSO (4%)	–	1	2	1	1.33 ± 0.58	–
		+	1	2	2	1.67 ± 0.58	–
Positive control (2NF + 4NQNO)	2NF (2) + 4NQNO (0.5)	–	48	48	48	48.00 ± 0.00**	25.13
Positive control (2AA)	2AA (5)	+	48	48	48	48.00 ± 0.00**	21.42

2NF, 2-Nitrofluorene; 4NQNO, 4-nitroquinoline-*N*-oxide; 2AA, 2-aminoanthracene.
** $p < 0.05$.

use in medicine (Fabricant and Farnsworth, 2001). Natural products, as either pure compounds or standardized plant extracts, provide variety of therapeutic opportunities. However, physicians, pharmacists and consumers are besieged with questions about the safety and efficacy of herbal remedies (Cheng et al., 2009).

Licorice root has a long history of use in both Eastern and Western cultures as a remedy in the treatment for a wide range of ailments. It is one of the oldest and most popular herbal medicines in the world, and is recorded in the pharmacopoeias of many Asian and European countries including China (CP, 2005), Japan (JP, 1996), the United Kingdom (BP, 1998). *G. glabra* is also listed in the US (USP, 2007) and European (EP, 1997) pharmacopeias. Many studies have reported the pharmacological efficacy and benefits of *G. glabra*, but little information has been reported about its risk and safety.

Fenwick et al. (1990) stated that, approximately 90% of the US licorice supply is used by the tobacco industry, with the remainder split evenly between the food and pharmaceutical industries. There are several forms of licorice extracts are sold worldwide. Significantly, glycyrrhizin (glycyrrhizic acid; glycyrrhizinate) constitutes 10–25% of licorice root extract and is considered as primary active ingredient. However, consumption of glycyrrhizin as dietary supplement is limited due to some side effects. There is abundant evidence from both case reports and clinical studies that the habitual consumption of glycyrrhizin results in adverse effects marked by the development of pseudohypercorticosteroidism (Isbrucker and Burdock, 2006). Dominant lethal testing in male rats suggested that an intake of 4000–5000 mg glycyrrhizin/kg/day could lead to mutagenic effects in offspring (Sheu et al., 1986; SRI, 1977).

Due to reported side effects, Schulz et al. (1998) suggested an average daily dose not to exceed 5–15 g of dried root (equivalent to 200–600 mg glycyrrhizin) for the treatment of gastrointestinal ailments, with a recommended course of treatment not exceeding 4–6 weeks.

Based on the above considerations, licorice extract (GutGard™) was prepared to contain negligible amount of glycyrrhizin. Glycyrrhizin is removed to the greater extent from the extract by

Table 4A
Chromosomal aberration test with GutGard™ – short term (4 h) with S9.

Treatment	Vehicle DMSO (1%)		Benzo [a] pyrene (20 µg/mL)		GutGard™ (4 µg/mL)		GutGard™ (12.6 µg/mL)		GutGard™ (40 µg/mL)	
Replicate	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
Total no. metaphases	100	100	100	100	100	100	100	100	100	100
Normal	100	97	75	46	100	100	100	100	99	100
Chromatid break	–	1	5	19	–	–	–	–	1	–
Chromosome break	–	1	6	11	–	–	–	–	–	–
Deletion	–	–	–	–	–	–	–	–	–	–
Ring	–	–	1	2	–	–	–	–	–	–
Dicentric	–	1	13	22	–	–	–	–	–	–
Total aberrations	0	3	25	54	0	0	0	0	1	0
Total aberrations[a] (Mean ± SD)	1.50 ± 2.10		39.50 ± 20.50*		0.00 ± 0.00		0.00 ± 0.00		0.50 ± 0.70	
Mitotic index[b] (Mean ± SD)	5.80 ± 0.40		2.80 ± 0.20**		5.08 ± 0.70		5.30 ± 0.80		2.20 ± 0.90**	

R1, Replicate 1; R2, Replicate 2.
[a] Total aberrations (mean ± standard deviation from 200 metaphases per treatment).
[b] Mitotic index (mean ± standard deviation from 2000 total cells per treatment).
* Significantly increased compared to the vehicle control ($p < 0.05$).
** Significantly decreased compared to the vehicle control ($p < 0.05$).

Table 4B
Chromosomal aberration test with GutGard™ – short term (4 h) without S9.

Treatment	Vehicle DMSO (1%)		EMS (500 µg/mL)		GutGard™ (4 µg/mL)		GutGard™ (12.6 µg/mL)		GutGard™ (40 µg/mL)	
Replicate	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
Total no. metaphases	100	100	100	100	100	100	100	100	100	100
Normal	94	98	74	75	98	97	99	98	96	99
Chromatid break	4	1	19	16	–	2	1	1	2	1
Chromosome break	2	1	4	8	–	–	–	–	–	–
Deletion	–	–	–	–	–	–	–	–	–	–
Ring	–	–	–	1	–	–	–	1	2	–
Dicentric	–	–	–	–	2	1	–	–	–	–
Total aberrations	6	2	26	25	2	3	1	2	4	1
Total aberrations[a] (Mean ± SD)	4.00 ± 2.80		25.50 ± 0.70*		2.50 ± 0.70		1.50 ± 0.70		2.50 ± 2.10	
Mitotic index[b] (Mean ± SD)	4.70 ± 1.00		3.20 ± 0.60**		3.80 ± 0.50		3.70 ± 0.40		2.70 ± 0.30**	

R1, Replicate 1; R2, Replicate 2.
[a] Total aberrations (mean ± standard deviation from 200 metaphases per treatment).
[b] Mitotic index (mean ± standard deviation from 2000 total cells per treatment).
* Significantly increased compared to the vehicle control ($p < 0.05$).
** Significantly decreased compared to the vehicle control ($p < 0.05$).

Table 4C
Chromosomal aberration test with GutGard™ – long term (18 h) without S9.

Treatment	Vehicle DMSO (1%)		EMS (500 µg/mL)		GutGard™ (1.46 µg/mL)		GutGard™ (4.6 µg/mL)		GutGard™ (14.6 µg/mL)	
Replicate	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
Total no. metaphases	100	100	100	100	100	100	100	100	100	100
Normal	98	100	39	51	100	100	99	99	99	98
Chromatid break	1	–	37	32	–	–	1	–		
Chromosome break	–	–	18	14	–	–	–	1		1
Deletion	–	–	4	2	–	–	–	–		
Ring	–	–	1	–	–	–	–	–		1
Dicentric	1	–		–	–	–	–	–	1	
Total aberrations	2	0	61	49	0	0	1	1	1	2
Total aberrations[a] (Mean ± SD)	1.00 ± 1.40	55.00 ± 8.50*	0.00 ± 0.00	1.00 ± 0.00	1.50 ± 0.70					
Mitotic index[b] (Mean ± SD)	4.40 ± 0.70	2.50 ± 0.60**	4.00 ± 0.20	3.80 ± 0.30	2.70 ± 0.90**					

R1, Replicate1; R2, replicate 2.
[a] Total aberrations (mean ± standard deviation from 200 metaphases per treatment).
[b] Mitotic index (mean ± standard deviation from 2000 total cells per treatment).
* Significantly increased compared to the vehicle control ($p < 0.05$).
** Significantly decreased compared to the vehicle control ($p < 0.05$).

extracting the licorice roots with acetone (Chandrasekaran et al., 2011). GutGard™ is standardized to contain bio active constituents like glabridin, glabrol, isoliquiritigenin, eicosanyl caffeate and docosyl caffeate. Recent findings of the randomized double-blind, placebo-controlled, clinical trial on GutGard™, the root extract of *G. glabra*, revealed significant decrease in symptoms scores in

Table 5A
Percent micronucleated binucleated cells in short term (4 h) with S9, short term (4 h) without S9, long term (18 h) without S9.

Treatment	%MN-BN (R1)	%MN-BN (R2)	Total MN-BN (%)[a]
Short term with S9			
Vehicle – DMSO (1%)	0.10	0.10	0.10 ± 0.00
GutGard™ (4 μg/mL)	0.05	0.24	0.15 ± 0.13
GutGard™ (12.6 μg/mL)	0.05	0.29	0.17 ± 0.17
GutGard™ (40 μg/mL)	0.44	0.25	0.34 ± 0.13
Benzo [a] pyrene (20 μg/mL)	3.10	3.00	3.05 ± 0.07*
Short term without S9			
Vehicle – DMSO (1%)	0.40	0.80	0.60 ± 0.28
GutGard™ (4 μg/mL)	0.64	0.88	0.76 ± 0.17
GutGard™ (12.6 μg/mL)	0.68	0.68	0.68 ± 0.00
GutGard™ (40 μg/mL)	0.90	0.64	0.77 ± 0.18
EMS (400 μg/mL)	1.57	2.4	1.98 ± 0.59*
Long term without S9			
Vehicle – DMSO (1%)	0.69	0.34	0.52 ± 0.25
GutGard™ (1.4 μg/mL)	0.68	0.74	0.71 ± 0.05
GutGard™ (4.6 μg/mL)	0.10	0.40	0.25 ± 0.21
GutGard™ (14.6 μg/mL)	0.14	0.15	0.15 ± 0.01
EMS (400 μg/mL)	5.25	5.35	5.30 ± 0.07*

R1, Replicate 1; R2, Replicate 2.
[a] Percent micronucleated binucleated cells from 1000 cells per replicate; 2000 total cells per treatment for the study.
* Significantly increased compared to the concurrent vehicle control ($p < 0.05$).

concordance with improvements in almost all individual symptoms and found to be superior to placebo group in the management of functional dyspepsia (Raveendra et al., 2011). Also our previous *in vitro* studies confirmed its anti-inflammatory activity by inhibiting pro-inflammatory mediators, COX and LOX products (Thiyagarajan et al., 2011; Chandrasekaran et al., 2011).

Besides the possible use of GutGard™ as a therapeutic agent, knowledge about its genotoxic potential is also of interest from the point of view of human consumption. The use of *in vitro* assays was decisive in order to obtain a picture of the genotoxic potential of this plant product.

To evaluate the potential genotoxicity of GutGard™, Ames II™ tests, chromosome aberration test and a micronucleus test were employed. Ames II™ tests results indicated no significant increase in the number of revertant colonies in TA98 and TAMix upon treatment with GutGard™ regardless of the metabolic activation system. GutGard™ did not induce structural chromosome aberrations and micronucleus formation in the presence and absence of metabolic activation in CHO-K1 cell line. Nakagawa et al. (2008) demonstrated non mutagenic effect of licorice extract (Licorice flavonoid oil – LFO) in bacterial reverse mutation assay and *in vivo* micronucleus tests. However, LFO, concentration range of 600–700 μg/mL with S9 produced statistically significant increase in the occurrence of chromosome aberrations and cell toxicity in Chinese Hamster Lung cell. Interestingly, in our experiment GutGard™ showed 50% cytotoxicity to CHO-K1 cells at low concentrations (40 μg/mL) and also did not show any significant increase of chromosome aberration in the presence of S9. The difference in the results might be due to the difference in phytochemical presence of the licorice preparations.

Table 5B
Cytokinesis blocked proliferation index (CBPI) values in short term (4 h) with S9, short term (4 h) without S9, long term (18 h) without S9.

Treatment	CBPI (R1)	CBPI (R2)	Total CBPI[a]
Short term with S9			
Vehicle – DMSO (1%)	1.92	1.94	1.93 ± 0.01
GutGard™ (4 μg/mL)	1.86	2.06	1.96 ± 0.01
GutGard™ (12.6 μg/mL)	1.90	1.90	1.90 ± 0.00
GutGard™ (40 μg/mL)	1.45	1.42	1.44 ± 0.02**
Benzo [a] pyrene (20 μg/mL)	1.92	1.94	1.93 ± 0.01
Short term without S9			
Vehicle – DMSO (1%)	2.21	2.09	2.15 ± 0.08
GutGard™ (4 μg/mL)	2.23	2.19	2.21 ± 0.03
GutGard™ (12.6 μg/mL)	2.19	2.17	2.18 ± 0.01
GutGard™ (40 μg/mL)	1.56	1.55	1.56 ± 0.01**
EMS (400 μg/mL)	1.95	1.92	1.94 ± 0.02 **
Long term without S9			
Vehicle – DMSO (1%)	2.09	1.95	2.02 ± 0.10
GutGard™ (1.4 μg/mL)	2.14	2.12	2.13 ± 0.01
GutGard™ (4.6 μg/mL)	2.02	2.02	2.02 ± 0.00
GutGard™ (14.6 μg/mL)	1.59	1.57	1.58 ± 0.01**
EMS (400 μg/mL)	1.87	1.85	1.86 ± 0.01**

R1, Replicate 1; R2, Replicate 2.
[a] Cytokinesis blocked proliferation index 1000 cells per replicate, 2000 total cells per treatment for the study.
** Significantly decreased compared to the concurrent vehicle control ($p < 0.05$).

Several studies have reported the non-genotoxic effect of licorice extract and its constituents viz., glycyrrhizin, 18 alpha glycyrrhetic acid and 18 beta glycyrrhetic acid in *S. typhimurium* strains (Yamaguchi and Watanabe, 1984; Mitscher et al., 1986; Zani et al., 1993). Based on series of toxicity, genotoxic and teratologic studies, glycyrrhizin containing licorice root was reported safe for consumption (Isbrucker and Burdock, 2006).

There are limited reports pertaining to anti-mutagenic effect of licorice constituents. In a recent publication, isoliquitrin apioside, isolated from *G. glabra* was reported to show anti-genotoxic effect against hydrogen peroxide and 4NQNO induced genotoxicity in *Escherichia coli* PQ37 (Kaur et al., 2009). Pre-treatment with glycyrrhizic acid (GA), a sweetening component of licorice roots reduced cisplatin induced DNA fragmentation, micronucleus formation, and the kidney toxicity markers BUN, creatinine, and LDH and restoration of normal kidney histology (Arjumand and Sultana, 2011).

Kowsalya et al. (2011) demonstrated the antigenotoxic potential of glycyrrhetinic acid (pentacyclic triterpenoid derivative and the active aglycone of glycyrrhizin) against DMBA induced genotoxicity. Glycyrrhetinic acid (GA) also prevented DNA damage and unscheduled DNA synthesis induced by benzo (alpha) pyrene in Chinese hamster lung cell line (Chen and Han, 1994).

Only one researcher reported a positive mutagenic response on licorice extract in *S. typhimurium* TA100 strain, but not in TA98. However, this response was not clearly concentration-dependent

suggesting either some toxicity or influence on the DNA repair mechanisms at the higher concentrations (Martinez et al., 1999).

GutGard™, a flavonoid rich extract containing insignificant amount of glycyrrhizin was not mutagenic to TA98 and TAMix strains of *S. typhimurium*, and did not induce or increase the incidence of structural chromosome aberrations or micronucleus formation in CHO-K1 cells.

5. Conclusion

The results of battery of genotoxicity models clearly confirmed that GutGard™ was devoid of genotoxic effect under our experimental conditions.

Conflict of interest statement

All authors declare that they have no conflicts of interest.

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