Modulation of genotoxicity of oxidative mutagens by glycyrrhizic acid from *Glycyrrhiza glabra* L.

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ABSTRACT

Background: The chemopreventive effects of certain phytoconstituents can be exploited for their use as functional foods, dietary supplements and even as drugs. The natural compounds, acting as anti-genotoxic and free radical scavenging compounds, may serve as potent chemopreventive agents. These can inhibit DNA modulatory activities of mutagens and help preventing pathological processes. **Objectives:** Present study on *Glycyrrhiza glabra* L., a promising medicinal plant, widely used in traditional medicine, focused on the bioassay-guided fractionation of its extracts for the isolation of certain phytochemicals with anti-genotoxic potential against oxidative mutagens. **Materials and Methods:** The methanol extract of *Glycyrrhiza glabra* rhizomes was subjected to column chromatography, and isolated fraction was evaluated for its anti-genotoxic and antioxidant potential using SOS chromotest, Comet assay, and DPPH radical scavenging assay. **Results:** GLG fraction, which was characterized as Glycyrrhizic acid, inhibited the genotoxicity of oxidative mutagens *viz.,* H_2O_2 and 4NQOquite efficiently. In SOS chromotest, using *E.coli* PQ37 tester strain, it inhibited induction factor induced by H_2O_2 and 4NQO by 75.54% and 71.69% at the concentration of 121.46 µM,respectively. In Comet assay, it reduced the tail moment induced by H_2O_2 and 4NQO by 70.21% and 69.04%, respectively, at the same concentration in human blood lymphocytes. The isolated fraction also exhibited DPPH free radical scavenging activity and was able to scavenge 85.95% radicals at a concentration of 120 µM. **Conclusion:** Glycyrrhizic acid is a potential modulator of genotoxins as well as efficient scavenger of free radicals.

Key words: Chemoprevention, *Glycyrrhiza glabra* L., glycyrrhizic acid, H_2O_2, oxidative mutagens, 4NQO

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INTRODUCTION

Environmental pollution has introduced certain xenobiotic compounds, which act as mutagens and carcinogens as well as sources of reactive oxygen species production in various life forms. Research in the field of search for natural anti-mutagenic/antioxidant compounds has gained pace in the last few decades as the synthetic compounds often come with certain undesirable side-effects. Plant secondary metabolites are known to possess a wide range of pharmacological properties like acetylcholinesterase inhibitory effects, anti-microbial, anti-inflammatory, and anti-diabetic activities.[1,2] Similarly, several natural compounds exhibit anti-carcinogenic or anti-mutagenic activity against environmental carcinogens and mutagens.[3] The agents, which are able to interfere with mutation, have the potential to interfere with early stages of cancer. Various natural compounds, evaluated for chemopreventive potential, have been found to regulate cellular signaling of proliferation and death, and thus conferring a preventive benefit to the host.[4]

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Glycyrrhiza glabra L. (Fabaceae), commonly known as 'Licorice,'is considered as the oldest and most widely used herbal drugs around the world. Traditionally, it is used for its anti-viral effects as well as for respiratory, gastrointestinal, cardiovascular, genitourinary, eye and skin disorders.[5] The antioxidant, anti-genotoxic and anti-inflammatory activities of *G. glabra* leaves have been documented.[6] The anti-ulcerogenic action[7] as well as the anti-ulcer[8] of standardized extract of *G. glabra* has also been reported. The major bioactive constituents of *G. glabra* consists of flavonoids and pentacyclic triterpene saponins, which include isoliquiritigenin, glycyrrhizin, and glabridin.[9] The

present study aimed to isolate potent anti-genotoxic/antioxidant phytoconstituents from *G. glabra* through bioactivity-guided fractionation and to evaluate for their protective effect against DNA damage induced by oxidative mutagens *viz.*, hydrogen peroxide (H_2O_2) and 4-Nitroquinoline 1-oxide (4NQO) by employing SOS chromotest and Comet assay and evaluating their free radical scavenging activity.

MATERIALS AND METHODS

Plant material

The rhizomes of *G. glabra* were purchased from a local market at Amritsar, India. Voucher specimen No. 0342-A-03/2006 (*G. glabra*) has been deposited in the herbarium of the Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India.

Chemicals

Ethidium bromide, ortho-nitrophenyl-β-D-galactopyranoside (ONPG), nicotinamide adenine dinucleotide phosphate, (NADP), glucose-6-phosphate (G6P), normal melting point agarose (NMPA), and low melting point agarose (LMPA) were purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India. Histopaque 1077 from Sigma Chemicals (St. Louis, MO, USA); *para*-nitrophenyl phosphate (PNPP) from Sisco Research Laboratories Pvt. Ltd.; polyethyleneglycol-4-tetraoctylphenolether (Triton X-100), hydrogen peroxide, dimethyl sulphoxide were procured from Qualigens Fine Chemicals, Mumbai, India. *Escherichia coli* PQ37 strain was purchased from Institute Pasteur, France. All the chemicals used were of analytical grade.

Extraction/fractionation of rhizomes of *Glycyrrhiza glabra* L.

The rhizomes of *G. glabra* (1 kg) were washed and dried in oven at 40°C. The dried material was powdered and extracted 5 times with 80% methanol each time (3 liter ×5). The extract was concentrated using rotary evaporator (Buchi) and was further lyophilized on a lyophilizer to get the methanol extract (MeOH-GG), which was subjected to dry column chromatography. Dry column chromatography of MeOH-GG yielded fractions recovered from chloroform: Methanol (75/25 fractions) and (0/100 fractions), which were pooled, dried, and after making slurry, were subjected to reverse phase column chromatography by using silica gel RP-18, (running solvent mixture of chloroform: EtOAc: Methanol: 40:40:20). Five fractions were collected (each of 100 ml). The fourth fraction yielded a crystalline compound named as 'GLG' after placing the liquid fraction at 4°C in refrigerator [Figure 1].

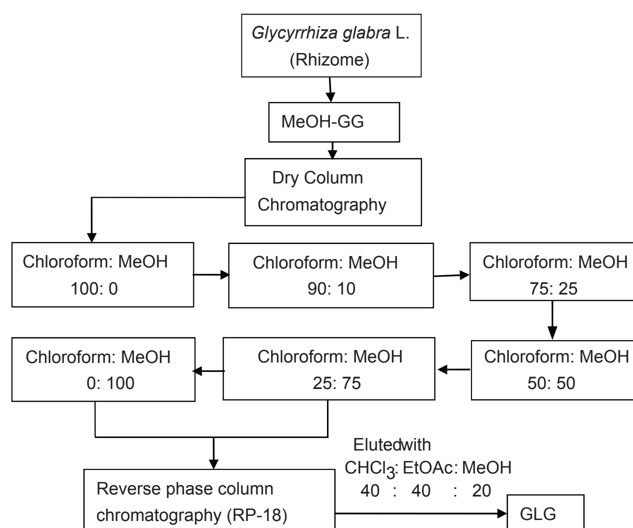

Figure 1: Schematic representation of isolation of GLG fraction from methanol extract of *G. glabra* L

SOS Chromotest

The ability to inhibit bacterial genotoxicity was tested in *Escherichia coli* PQ37 using SOS chromotest.[10] Hydrogen peroxide and 4NQO were used as mutagens to induce SOS inducing potency (SOSIP). Culture of *E. coli* PQ37 was grown at 37°C in Luria broth medium (1% bactotryptone, 0.5% yeast extract, 1% NaCl, and 20 μg/ml ampicillin) and diluted 1:9 into fresh medium subsequently. Aliquots of 100 μl were distributed into test tubes containing different concentrations of GLG fraction, making final volume to 0.6 ml. A positive control was prepared by exposure of the bacteria to either H_2O_2 or 4NQO. After 2 h of incubation at 37°C, with shaking, 300 μl samples were used for assay of β-galactosidase (β-gal) and alkaline phosphatase (Ap) activities. The different concentrations of GLG fraction samples were also tested in the absence of mutagens for their genotoxic effect. The induction factor (IF) was calculated as the ratio of Rc/Ro where Rc is equal to β-galactosidase activity/alkaline phosphatase activity determined for the test compound at concentration c, and Ro is equal to β-galactosidase activity/alkaline phosphatase activity in the absence of test compound. In the SOS chromotest assay, the compounds are classified as non-genotoxic, if the induction factor (IF) remains <1.5 and genotoxic, if IF exceeds 2.0. Anti-genotoxicity was expressed as percentage inhibition of genotoxicity according to the formula:

$$\text{Inhibition } (\%) = 100 - (IF_1 - IF_0 / IF_2 - IF_0) \times 100$$

Where,

IF_1 is the induction factor in the presence of the test compound and mutagen (H_2O_2/4NQO)

IF_2 the induction factor in the absence of the test compound (only mutagen) and

IF_0 is the induction factor of the blank.
Data was collected (mean±standard error) of triplicate experiments.

Comet assay

The alkaline comet assay was performed using human blood lymphocytes.[11] Heparinized blood samples were obtained by venipuncture from non-smoking, healthy male donor aged 25-40 years. Lymphocytes were isolated by the method of Boyum.[12] The blood was mixed with equal volume of phosphate buffer saline (PBS; pH 7.2). This mixture was then overlaid to double volume of histopaque 1077 and centrifuged at 1500 rpm for 20 min. White ring of lymphocytes, formed at the interface of plasma and histopaque, was aspirated very carefully with the help of pasteur pipette. The lymphocytes were then diluted in PBS and centrifuged at 2000 rpm for 15 min. The supernatant was discarded, and pellet was again suspended in PBS and centrifuged at 2000 rpm for 15 minutes. This step was repeated twice to remove the impurities from lymphocytes. Isolated lymphocytes were stored in heparinized eppendorf tubes at 4°C and their viability was determined by trypan blue dye exclusion analysis.[13]

Comet assay was performed with lymphocytes suspended in 1 ml PBS and incubated for 30 min at 37°C with 20 μl of mutagen (H_2O_2/4NQO) in the presence 20 μl of different concentrations of GLG fraction. Images of 100 randomly selected cells from each sample were obtained using an Epifluorescent Nikon microscope connected with a digital camera. Extent of DNA damage was evaluated in terms of Tail moment (an index of DNA damage, which considers both tail length and the fraction of DNA in comet tail) by analyzing images on a computerized image analysis system (Lucia Comet Assay Software 4.8 of Laboratory Imaging Ltd.). To check for toxicity or an effect on DNA, GLG fraction was tested alone (without mutagen) also. The percentage inhibition was calculated by the formula:

$$\text{Inhibition (\%)} = (T_1 - T_C)/(T_1 - T_0) \times 100$$

Where,
T_1=Tail moment induced by H_2O_2/4NQO
T_C=Tail moment of different concentrations of GLG fraction in presence of H_2O_2/4NQO
T_0=Tail moment of negative control

The whole experiment was repeated thrice to attain significant data.

DPPH radical scavenging assay

The effect of GLG fraction on DPPH radical was estimated according to the method given by Blois.[14] 300 μl of different concentrations of GLG fraction were added in 2 ml of DPPH (0.1 mM methanolic solution). The mixture was shaken well, placed at room temperature for 15 min., and absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The percent DPPH decolorization of the sample was calculated by the equation:

$$\text{Radical scavenging activity} = \frac{A_0 - A_1}{A_0} \times 100$$

Where,
A_0 is the absorbance of DPPH solution.
A_1 is the absorbance of reaction mixture (containing test compound and DPPH solution)

Statistical analysis

The results are presented as the average and standard error of experiments performed in triplicates. The data were analyzed for statistical significance using analysis of variance (one-way ANOVA), and difference among average values was compared by high-range statistical domain (HSD) using Tukey's Test.[15] The statistical significance was checked at $P<0.05$. IC_{50} was calculated from the regression line.

RESULTS

Characterization of GLG fraction

The fraction 'GLG' isolated from MeOH-GG through reverse phase column chromatography was characterized using ^1H NMR, ^{13}C NMR, Distortionless Enhancement by Polarization Transfer (DEPT). TLC of this fraction gave one spot at Rf 0.28 comparable to that of standard glycyrrhizic acid in the solvent system *n*-butanol: Distilled water: Acetic acid:: 7: 2:1. GLG fraction was further analyzed by electrospray ionization tandem mass spectrometry (ESI-QTOF-MS/MS) in positive ion mode. ESI-MS of GLG fraction generated two major molecular ion peaks at m/z 823 $[M+H]^+$. Further mass fragmentation of m/z 823 generated the two major fragments at m/z 647 and 471 $[\text{aglycone}+H]^+$ due to the sequential glycosidic cleavage of two glucouronic units (176 U). The fragment at m/z 453 $[\text{aglycone}+H-H_2O]^+$ was observed due to the loss of one H_2O unit from the aglycone part. Therefore, on the basis of mass spectral data and comparison with standard glycyrrhizic acid, the GLG fraction was identified as 'Glycyrrhizic acid' (GA) [Figure 2].

Anti-genotoxic potential

In the SOS chromotest assay, induction factor of different concentrations of GA remained below 1.5, revealing that it was not genotoxic to the indicator bacteria. The doses of 1.0 mM of H_2O_2 and 20 μg/ml of 4NQO

induced a significant SOS response without affecting the alkaline phosphatase activity. GA inhibited IF caused by H_2O_2 and 4NQO by 75.54% and 71.69%, respectively, at a concentration of 121.46 μM. The fraction showed moderate reduction in induction factor, even at lower test doses [Table 1].

In Comet assay, GA dose dependently inhibited the induced DNA damage in human blood lymphocytes. Among the viability test, the lymphocytes showed a viability ranging from 93% to 97%. The GA, at a concentration of 3.78 μM, inhibited the H_2O_2-induced DNA damage by reducing the tail moment by 11.04%, which dose-dependently increased up to 70.21% at concentration 121.46 μM [Figure 3]. A dose-response relationship showed significance at $P \leq 0.05$. The Tail moment induced by 4NQO was reduced by 69.04% at maximum concentration tested [Figure 4].

Antioxidant activity

The ability of compounds to donate hydrogen ion to stabilize the DPPH radical is a measure of their antioxidant activity. GA showed a very strong activity by effectively scavenging the free radicals by 85.96% at 120 μM concentration. The dose-response relationship was observed, and a logarithmic behavior was followed by the concentrations used for the present study at $P \leq 0.05$ [Figure 5].

DISCUSSION

Dietary compounds, including vitamins, minerals, carotenoids, and a large class of phytochemicals, have been acknowledged as potential chemopreventive agents. These prevent carcinogens to hit their cellular targets by various mechanisms as scavenging reactive oxygen species and enhancing DNA repair. Triterpenoids, a large and diverse group of naturally-occurring compounds, possess important pharmacological properties. These have been reported to exhibit anti-cancer[16] and anti-viral[17] and anti-HIV[18] activities. Triterpenoids isolated from methanolic extract of *Eucalyptus camaldulensis* Dehnh. fruits have been found to inhibit proliferation of the A2780 human ovarian cancer cell line.[19] Triterpenoids from the roots of *Astilbe myriantha* Diels have found to be anti-fungal agents.[20]

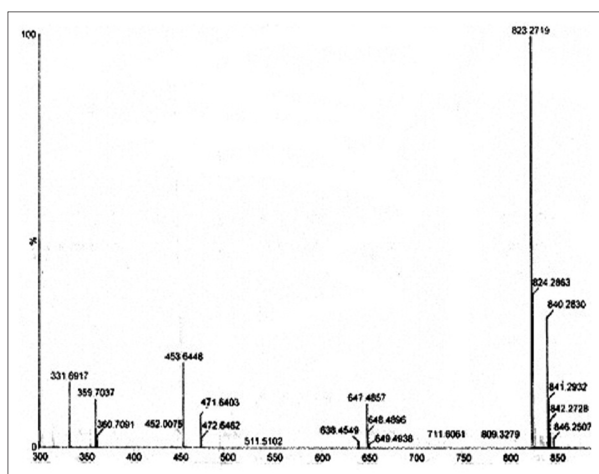

Figure 2: ESI-QTOF-MS/MS of GLG fraction isolated from *G. glabra* L

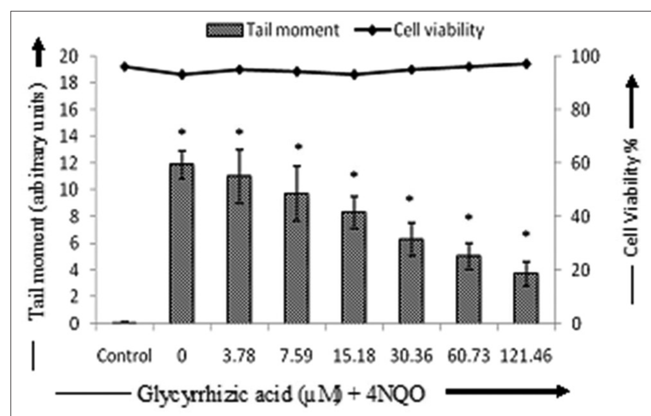

Figure 4: Decrease in tail moment by Glycyrrhizic acid from *G. glabra*. in human blood lymphocytes in Comet assay. Level of statistical significance; $P \leq 0.05$ compared to positive control (4NQO)

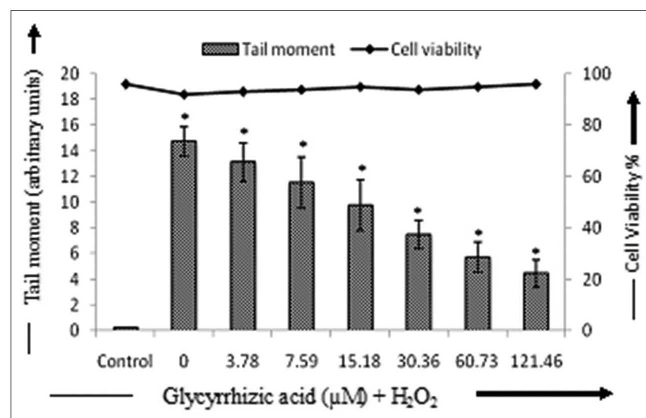

Figure 3: Decrease in tail moment by Glycyrrhizic acid from *G. glabra*. in human blood lymphocytes in Comet assay. Level of statistical significance; $P \leq 0.05$ compared to positive control (H_2O_2)

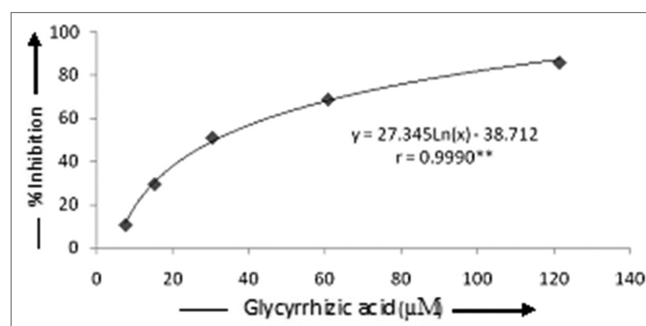

Figure 5: Effect of Glycyrrhizic acid isolated from *G. glabra*in DPPH assay. **$P \leq 0.05$

Table 1: Effect of Glycyrrhizic acid from *G. glabra* L. on genotoxicity of H_2O_2 and 4NQO in SOS chromotest using *E. coli* PQ37 tester strain

Treatment	Dose (μM)	β-Galactosidase units Mean±SD	Alkaline phosphatase units Mean±SD	Induction factor (IF)	Percent inhibition
Positive Controls					
H_2O_2	1 mM	4.48±0.02	12.98±0.11	7.50	–
4NQO	20 μg/ml	4.26±0.01	13.11±0.21	7.04	–
Negative Control	0.00	0.60±0.03	13.00±0.20	1.00	–
	3.78	0.69±0.02	13.00±0.39	1.15	–
	7.59	0.67±0.03	12.99±0.09	1.12	–
	15.18	0.66±0.06	12.98±0.22	1.10	–
	30.36	0.67±0.04	13.01±0.22	1.12	–
	60.73	0.68±0.06	13.05±0.21	1.13	–
	121.46	0.67±0.04	13.05±0.23	1.12	–
H_2O_2+GA	3.78	4.12±0.05	13.02±0.13	6.86*	09.85
	7.59	3.88±0.10	13.02±0.15	6.41*	16.77
	15.18	3.30±0.08	13.09±0.11	5.48*	31.08
	30.36	2.59±0.05	13.03±0.09	4.30*	49.24
	60.73	2.12±0.10	13.09±0.05	3.50*	61.54
	121.46	1.55±0.08	13.00±0.07	2.59*	75.54
4NQO+GA	3.78	4.02±0.11	13.10±0.20	6.65*	06.06
	7.59	3.84±0.05	13.09±0.25	6.36*	11.26
	15.18	3.30±0.11	13.12±0.13	5.45*	26.33
	30.36	2.92±0.08	13.13±0.22	4.82*	36.76
	60.73	1.98±0.12	13.07±0.28	3.28*	62.26
	121.46	1.65±0.13	13.10±0.18	2.71*	71.69

SD = Standard deviation, Data shown are mean ± SD of three independent experiments, Level of statistical significance, *P<0.05 with respect to positive control

4NQO, a quinoline derivative, is reported to be tumorigenic to lung, esophagus, stomach, skin and other organs and also as a co-carcinogen in the liver.[21] 4-HAQO, the 4-electron reduction product of 4-NQO metabolism, is alleged to be a contiguous carcinogen as it reacts spontaneously with DNA in the presence of oxygen and can get metabolized to DNA-reactive esters.[22] H_2O_2 causes extensive oxidative damage as it interacts with DNA through highly reactive oxygen and radical species.[23]

In the present study, the glycyrrhizic acid (GA), a triterpenoid glycoside, isolated from *G. glabra*, very efficiently suppressed the 4NQO and H_2O_2 induced genotoxicity in both SOS and Comet assay, respectively. It accounts for the sweet taste of licorice root. Among the natural saponins, GA is a molecule composed of a hydrophilic part, two molecules of glucuronic acid, and a hydrophobic fragment, glycyrrhetic acid.[24]

In SOS chromotest, GA reduced the induction factor of H_2O_2 with IC_{50} 34.61 μM. Earlier, there are reports revealing that GA protected human hepatoma cell line against aflatoxin-induced oxidative stress[25] and the chemopreventive activity of GA on 12-O-tetradecanoyl phorbol-13-acetate-induced cutaneous oxidative stress and tumor promotion in Swiss albino mice.[26] In Comet assay, it reduced the tail moment induced by H_2O_2 with IC_{50} 34.95 μM. In another study, comet assay was utilized to evaluate DNA protection capability of GA and revealed that intraperitoneal administration of GA to mice protected cellular DNA from gamma-radiation-induced damage.[27] The ability of silver nanoparticles-glycyrrhizic acid complex (SN-GLY) to protect against ionizing radiation using Swiss albino mice was assessed by employing the comet assay. It was found that immediately after 4 Gy gamma radiation exposures, the treatment of mouse blood leucocytes with SN-GLY *ex vivo* enhanced the rate of repair of cellular DNA damages.[28] GA has also been reported to prevent the lung injuries induced by short term exposures of benzo(a)pyrene,[29] protecting SKH-1 hairless mice against UVB radiation-induced skin tumor formation[30] and acting as anti-hyperglycemic and anti-dyslipidemic compound.[31]

GA exhibited IC_{50} of 34.41 μM in scavenging DPPH free radicals, which is in agreement with the reports revealing GA to be potent scavenger of reactive oxygen species and with anti-inflammatory activity.[32,33] Further, there are studies indicating GA and 18 β-glycyrrhetinic acid to possess anti-inflammatory activity. Both suppressed the expression of pro-inflammatory genes via inhibition of NF-kB and PI3K activity and thus decreased the excessive generation of NO, PGE2, and ROS.[34] Administration of *G. glabra* polysaccharides to mice, which were fed high-fat diet, significantly enhanced immune and antioxidant enzyme activities as compared to the control mice.[35] Similarly, the protective effects of GA on oxidative injury induced by tert-butyl hydroperoxide (t-BHP) leading

to apoptosis in cultured primary rat hepatocytes have also been demonstrated. GA modulated the critical end points of apoptosis induced by oxidative stress and thus GA could be beneficial against liver diseases.[36] In our earlier studies, we have reported GA to be a significant inhibitor of COX-2. It showed 72.60% and 95.08% COX-2 inhibition at concentrations 1 μM and 10 μM, respectively.[37]

CONCLUSION

The present study provides an insight into the role of glycyrrhizic acid as a strong modulator of genotoxic oxidative mutagens and a potent scavenger of free radicals.

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