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In Vitro and *In Vivo* Genotoxicity Assessment of *Piper longum L.* Powder and Hot-Water Extract

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Abstract

Piper longum L. is used in traditional medicine, but genotoxicity evidence must be related to the preparation tested. This study evaluated P. longum powder (PL-P) and a freeze-dried hot-water extract (PL-W) using bacterial reverse mutation, *in vitro* chromosomal aberration, and mouse bone marrow micronucleus assays. Four chemical markers were quantified by high-performance liquid chromatography. The main bacterial experiment tested five strains at 12.346–1000 ≒g/plate with and without rat liver S9. Chinese hamster lung cells were treated for 6 h with or without S9 or for 22 h without S9. Male ICR mice received 500, 1000, or 2000 mg/kg by oral gavage on two consecutive days. All four marker contents were lower in PL-W than in PL-P; piperine contents were 0.414% and 2.427%, respectively. Neither preparation produced a biologically relevant bacterial mutagenic response or a statistically significant increase in structural chromosomal aberrations. Relative population doubling at the highest scored PL-P concentrations was 45–49%, and PL-W produced a value of 50% after prolonged exposure at 1600 ≒g/mL. No statistically significant increase in bone marrow micronuclei was detected, and both *in vivo* positive controls produced significant responses. These results provide preparation-specific evidence of no detected genotoxic response under the conditions tested. Precipitation limits interpretation of dissolved *in vitro* exposure, and bone marrow exposure was not demonstrated. The findings do not establish safety for long-term human use.

Keywords: Piper longum; Genotoxicity; Bacterial reverse mutation; Chromosomal aberration; Micronucleus assay

Introduction

Piper longum L. (Piperaceae), or long pepper, is used as a spice and in traditional medicine [1]. Its extracts and constituents have attracted interest for their immunomodulatory, antitumor, and other biological activities [2, 3]. However, pharmacological activity and a history of use do not establish the genotoxic safety of a

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defined preparation. Guidance for herbal products emphasizes the relationship between the material tested and the preparation being assessed [4].

Chemical characterization is therefore essential. Piperine content varies among *P. longum* populations and tissues [1], and chromatographic studies demonstrate variation in piperamide profiles and support multicomponent analysis [3, 5, 6]. Piperine can also inhibit human P-glycoprotein and CYP3A4 in experimental systems, illustrating its potential to affect biological exposure [7]. Powdered material and hot-water extracts should consequently be evaluated as distinct mixtures rather than assumed to be toxicologically interchangeable.

Evidence for individual constituents is informative but cannot replace testing of whole preparations. Negative bacterial and mouse micronucleus findings have been reported for piperine [8], and subsequent work found no micronucleus induction in Chinese hamster ovary cells or mice under the conditions examined [9]. Studies reporting protection by piperine or *P. longum* extracts against chemically induced genetic damage address a different question from intrinsic genotoxicity [10, 11]. Conversely, piperlongumine induced DNA damage in breast cell lines [12]. These findings emphasize the importance of chemical identity, exposure conditions, and the biological model when interpreting constituent-level evidence.

Complementary assays address different forms of genetic damage: bacterial reverse mutation detects gene mutations, *in vitro* chromosomal aberration testing detects structural chromosome damage, and the *in vivo* micronucleus assay detects chromosomal damage or mitotic disturbance in erythroblasts [13–15]. We therefore evaluated *P. longum* powder (PL-P) and a freeze-dried hot-water extract (PL-W) using these three assays and characterized their piperine, pellitorine, pipernonaline, and guineensine contents by HPLC. The objective was to assess their genotoxic potential under defined experimental conditions and provide preparation-specific evidence for nonclinical safety assessment.

Materials and Methods

Chemicals and reagents

Sodium azide (SA), 2-nitrofluorene (2-NF), 9-aminoacridine (9-AA), 4-nitroquinoline-N-oxide (4-NQO), benzo[*a*]pyrene (BP), 2-aminoanthracene (2-AA), mitomycin C (MMC), cyclophosphamide monohydrate (CPA), colchicine, dimethyl sulfoxide were obtained from Sigma-Aldrich (USA). Sterile distilled water was used as the vehicle control unless otherwise specified. Metabolic activation in the bacterial reverse mutation and *in vitro* chromosomal aberration assays was provided by a liver S9 fraction obtained from Aroclor 1254-induced male Sprague–Dawley rats (Molecular Toxicology, Inc., USA; cat. no. 11-01L). The S9 mix was prepared immediately before use by combining the S9 fraction with cofactors (Wako Pure Chemical Industries, Ltd., Japan; cat. no. 309-50611) and was kept chilled until use. Both *in vitro* assays included treatment conditions with and without metabolic activation.

Test articles and chemical characterization

Dried fruit spikes of *Piper longum* L. (Piperaceae) were authenticated by a botanical identification advisory committee through morphological examination based on the botanical origin and macroscopic characteristics described in the Korean Herbal Pharmacopoeia. Quality-control specifications and results for the starting material are summarized in Supplementary Table S1.

Suplementary Table S1: Quality control specifications and results for the *Piper longum* starting material.

Category	Analyte or test	Specification	Result
Heavy metal	Cd	≤0.3 ppm	-
	Pd as printed	≤5 ppm	-
	As	≤3 ppm	-
	Hg	≤0.2 ppm	-
Pesticide	Total BHC (α, β, γ)	≤0.2 ppm	Not detected
	Total DDT	≤0.1 ppm	Not detected
	Aldrin	≤0.01 ppm	Not detected
	Endrin	≤0.01 ppm	Not detected
	Dieldrin	≤0.01 ppm	Not detected
Sulfur dioxide	Residual SO_2	≤30 ppm	3 ppm
Specification	Loss on drying	≤10.0%	8.7%
	Ash	≤5.0%	4.6%
	Essential oil	≥0.2 mL	0.5 mL

Results refer to the herbal material used to prepare the powder and hot-water extract. Quality-control testing was performed according to the general test methods of the Korean Pharmacopoeia, 11th edition. Specifications are the acceptance criteria applied in this study. '-' indicates that no result was reported and does not denote non-detection. Total BHC comprises α-, β-, and γ-BHC; total DDT comprises p,p′-DDD, p,p′-DDE, o,p′-DDT, and p,p′-DDT. The metal designation "Pd" is retained as recorded. Essential oil specifications and results are expressed as volumes (mL); the test portion mass was not specified.

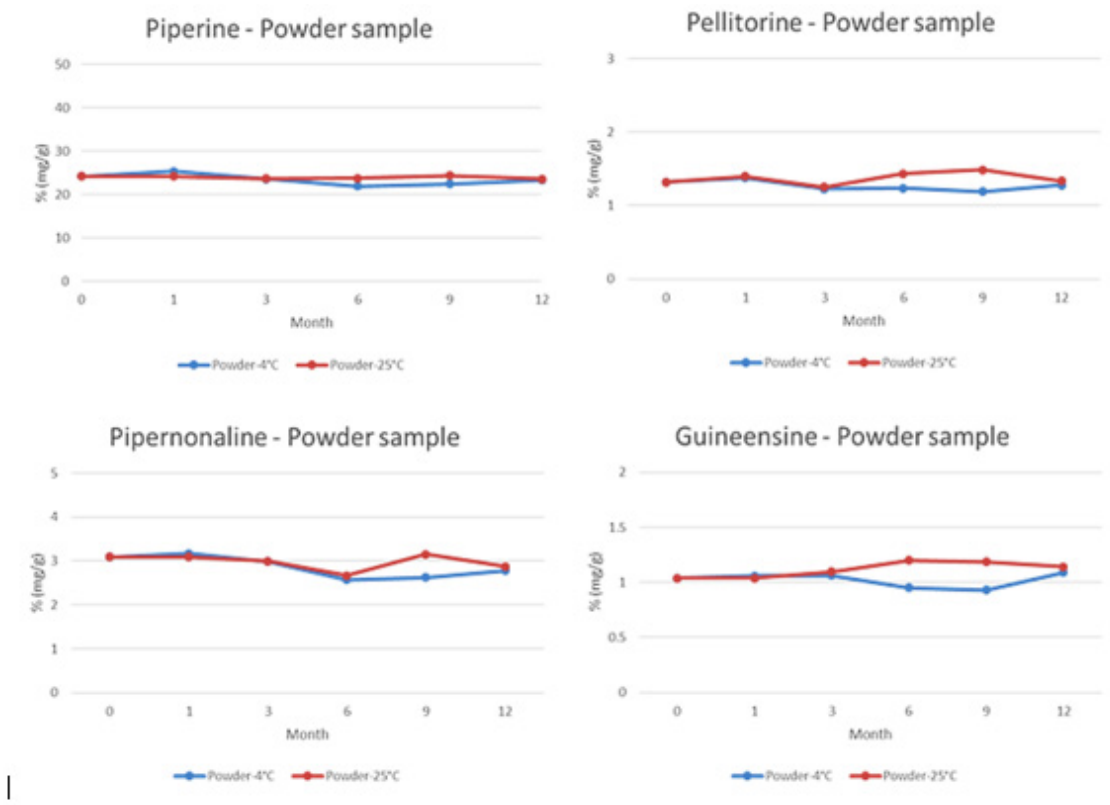
As, arsenic; BHC, benzene hexachloride; Cd, cadmium; DDD, dichlorodiphenyldichloroethane; DDE, dichlorodiphenyldichloroethylene; DDT, dichlorodiphenyltrichloroethane; Hg, mercury; ppm, parts per million.

Two test articles were prepared from the authenticated material: a powdered preparation (PL-P) and a hot-water extract (PL-W). The particle size of PL-P was assessed against the fine-powder specification of the Korean Pharmacopoeia. PL-W was prepared by hot-water extraction followed by freeze-drying. Sterile distilled water served as the vehicle for both preparations in the genotoxicity assays. Chemical characterization was performed by high-performance liquid chromatography (HPLC) to quantify four marker compounds: piperine, pellitorine, pipernonaline, and guineensine. Their contents and retention times are presented in Table 1. Marker contents were also measured at baseline and after 1, 3, 6, 9, and 12 months of storage at room temperature (25 °C) and under refrigeration. The corresponding profiles for PL-P and PL-W are presented in Supplementary Figures S1 and S2, respectively.

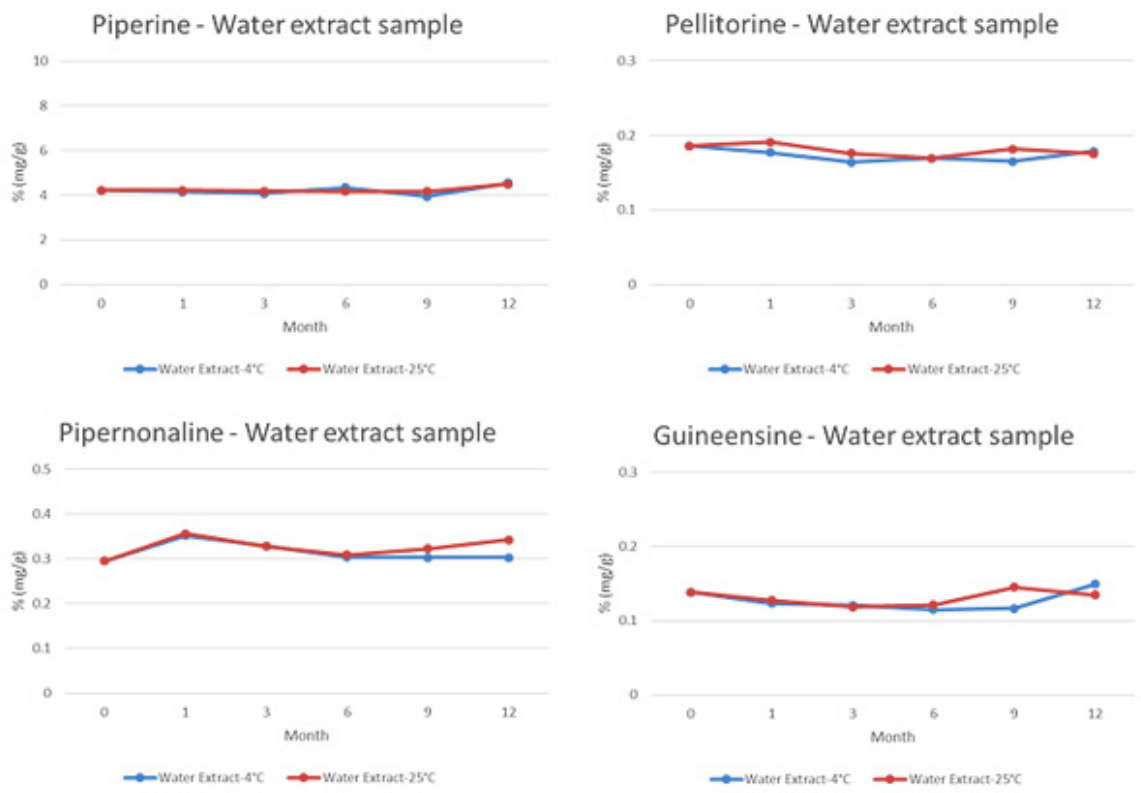
Table 1: Chemical marker contents of *Piper longum* powder and hot-water extract.

Marker	PL-P Content (%)	PL-W Content (%)	PL-P RT (min)	PL-W RT (min)
Piperine	2.427 ± 0.001	0.414 ± 0.001	37.28	37.38
Pellitorine	0.138 ± 0.001	0.008 ± 0.001	47.10	47.05
Pipernonaline	0.301 ± 0.001	0.021 ± 0.001	50.78	50.73
Guineensine	0.115 ± 0.001	0.008 ± 0.001	56.24	56.17

PL-P, *Piper longum* powder; PL-W, *Piper longum* hot-water extract; RT, retention time; HPLC, high-performance liquid chromatography.



Supplementary Figure S1: Chemical marker profiles during 12-month storage of Piper longum powder (PL-P). Contents of piperine (upper left), pellitorine (upper right), pipernonaline (lower left), and guineensine (lower right) were determined by high-performance liquid chromatography at baseline and after 1, 3, 6, 9, and 12 months of storage. Blue and red lines represent refrigerated storage and storage at 25 ^{0}C, respectively.



Supplementary Figure S2, Figure S3: Chemical marker profiles during 12-month storage of Piper longum hot-water extract (PL-W). Contents of piperine (upper left), pellitorine (upper right), pipernonaline (lower left), and guineensine (lower right) were determined by high-performance liquid chromatography at baseline and after 1, 3, 6, 9, and 12 months of storage. Blue and red lines represent refrigerated storage and storage at 25 °C, respectively.

Animals, ethical approval, and husbandry

Male specific-pathogen-free ICR mice (CrlOri:CD1; Orient Bio, Korea) were obtained at six weeks of age and acclimatized for six days before dosing. Animals were housed in polycarbonate cages (180 × 240 × 143 mm), with up to four mice per cage during acclimatization and up to three per cage during the pre-dosing and dosing periods. Room conditions were at 22 ± 3 °C and 40–75% relative humidity under a 12-hour light/dark cycle, with illumination of 150–300 lux and 10–20 air changes per hour. A standard laboratory diet (LabDiet #5053, PMI Nutrition International, USA) and filtered, ultraviolet-treated tap water were provided *ad libitum*. Mice were approximately seven weeks old at the start of treatment.

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Korea Institute of Toxicology. The approval numbers were 2007-0235 for the *Piper longum* powder (PL-P) study and 2007-0234 for the hot-water extract (PL-W) study.

The bacterial reverse mutation, *in vitro* chromosomal aberration, and mouse bone marrow micronucleus assays were conducted in compliance with Good Laboratory Practice (GLP).

Bacterial reverse mutation assay

The bacterial reverse mutation assay was performed using the plate-incorporation method [13, 16]. *Salmonella typhimurium* TA100, TA1535, TA98, and TA1537 and *Escherichia coli* WP2*uvr*A were obtained from Molecular Toxicology, Inc. (Boone, NC, USA). Strain characteristics, including amino acid requirements, UV sensitivity, the *rfa* phenotype, R-factor retention, and spontaneous reversion, were verified as applicable to each strain. Cultures were grown in 2.5% nutrient broth at 37 ± 1 °C with shaking at 120–200 rpm for approximately 10 h. Ampicillin was added for strains carrying pKM101. Bacterial suspensions were used at a density of at least 1×10^9 cells/mL. PL-P and PL-W were tested in the presence and absence of metabolic activation. Preliminary concentration-range-finding experiments evaluated 8, 40, 200, 1000, 3000, and 5000 μg/plate, with one plate per strain, concentration, and metabolic activation condition. Based on these experiments, the main assay evaluated 12.346, 37.037, 111.111, 333.333, and 1000 μg/plate, with four replicate plates per strain, concentration, and metabolic activation condition.

For each plate, 0.1 mL of test-article preparation or vehicle, 0.1 mL of bacterial suspension, and 0.5 mL of either 0.2 M sodium phosphate buffer or S9 mix containing 5% (v/v) S9 were mixed

with 2.0 mL of molten top agar maintained at approximately 45 °C. The top agar was supplemented with histidine and biotin for the *S. typhimurium* strains or tryptophan for *E. coli* WP2*uvr*A. The mixture was poured onto minimal glucose agar plates and allowed to solidify. Plates were then inverted and incubated at 37 ± 1 °C for approximately 48 h, after which revertant colonies were counted manually. Precipitation, turbidity, and changes in the bacterial background lawn were recorded.

Sterile distilled water served as the vehicle control for both test articles. Strain-specific positive controls were included under the corresponding metabolic activation conditions, as detailed in Tables 2 and 3. Additional treatments with 2-aminoanthracene (2-AA) in TA1535 and benzo[*a*]pyrene (BP) in TA98 were included without S9 to assess the dependence of their mutagenic responses on metabolic activation.

Supplementary Table S2: Concentration range-finding results for *Piper longum* powder (PL-P) in the bacterial reverse mutation assay

Concentration or control (µg/plate)	TA100	TA1535	TA98	TA1537	WP2*uvr*A
Without metabolic activation (−S9)					
0	136	10	32	14	15
8	145	8	32	14	9
40	153	10	31	13	12
200	149	11	34	14	17
1000	139	7	37	16	15
3000	136	10	32	12	21
5000	142	8	34	15	18
SA 0.5	228	192			
2-NF 2			412		
9-AA 50				428	
4NQO 1					516
2-AA 2‡		10			
BP 2‡			26		
With metabolic activation (+S9)					
0	147	7	40	7	23
8	147	10	42	5	22
40	132	8	32	11	21
200	142	8	43	11	25
1000	142	7	38	7	18
3000	143	6	44	8	29
5000	159	5	32	8	31
BP 2	580		368	194	
2-AA 2		204			
2-AA 4					253

Values represent revertant colony counts from one plate per strain, concentration, and metabolic activation condition. TA100, TA1535, TA98, and TA1537 are *Salmonella typhimurium* strains; WP2*uvr*A is an *Escherichia coli* strain. The concentration of 0 µg/plate denotes the vehicle control (sterile distilled water). Numbers accompanying control compounds indicate doses in µg/plate. −S9 and +S9 indicate the absence and presence of metabolic activation, respectively. '-' indicates that the control was not used for the corresponding strain.

‡ Additional treatments with 2-AA in TA1535 and BP in TA98 were conducted without S9 to assess the dependence of their responses on metabolic activation.

Precipitation was observed at ≥1000 **µg**/plate both upon mixing with top agar and after 48 h of incubation. Turbidity was observed at 5000 **µg**/plate after 48 h of incubation. These observations applied to all strains with and without S9. No bacterial toxicity was observed.

PL-W, *Piper longum* hot-water extract; S9, rat liver post-mitochondrial fraction used for metabolic activation; SA, sodium azide; 2-NF, 2-nitrofluorene; 9-AA, 9-aminoacridine; 4NQO, 4-nitroquinoline N-oxide; BP, benzo[*a*]pyrene; 2-AA, 2-aminoanthracene.

Table 2: Bacterial reverse mutation assay results for *Piper longum* powder (PL-P) with and without metabolic activation.

Concentration or control (μg/plate)	TA100	TA1535	TA98	TA1537	WP2*uvrA*
Without metabolic activation (–S9)					
0	123 ± 5	12 ± 3	22 ± 1	6 ± 1	13 ± 3
12.346	115 ± 5	12 ± 3	23 ± 4	6 ± 1	12 ± 1
37.037	135 ± 10	11 ± 1	25 ± 4	5 ± 1	14 ± 3
111.111	123 ± 9	12 ± 3	22 ± 3	6 ± 1	13 ± 2
333.333	112 ± 20	11 ± 2	20 ± 2	6 ± 2	15 ± 2
1000	128 ± 9	12 ± 3	22 ± 2	8 ± 1	18 ± 2
SA 0.5	346 ± 68	185 ± 18	-	-	-
2-NF 2	-	-	373 ± 40	-	-
9-AA 50	-	-	-	523 ± 127	-
4NQO 1	-	-	-	-	107 ± 13
2-AA 2‡	-	14 ± 1	-	-	-
BP 2‡	-	-	25 ± 8	-	-
With metabolic activation (+S9)					
0	126 ± 6	13 ± 2	36 ± 7	8 ± 1	15 ± 2
12.346	127 ± 5	12 ± 2	33 ± 5	7 ± 1	16 ± 2
37.037	130 ± 10	13 ± 3	35 ± 4	9 ± 2	16 ± 3
111.111	126 ± 6	13 ± 4	32 ± 2	7 ± 1	16 ± 2
333.333	127 ± 18	13 ± 2	33 ± 5	8 ± 1	14 ± 2
1000	121 ± 14	12 ± 1	35 ± 8	7 ± 1	14 ± 2
BP 2	882 ± 109		349 ± 5	132 ± 13	-
2-AA 2	-	240 ± 10	-	-	-
2-AA 4	-	-	-	-	129 ± 15

Values are mean revertant colony counts per plate (four plates per strain, concentration, and activation condition). The 0 μg/plate group is the sterile distilled water vehicle control; –S9/+S9 indicate absence/presence of metabolic activation. -, not tested for that strain. ‡ Additional 2-AA (TA1535) and BP (TA98) treatments without S9 assessed activation dependence.

PL-P, *Piper longum* powder; S9, rat liver post-mitochondrial fraction; SA, sodium azide; 2-NF, 2-nitrofluorene; 9-AA, 9-aminoacridine; 4-NQO, 4-nitroquinoline N-oxide; BP, benzo[*a*]pyrene; 2-AA, 2-aminoanthracene.

Table 3. Bacterial reverse mutation assay results for *Piper longum* hot-water extract (PL-W) with and without metabolic activation.

Concentration or control (μg/plate)	TA100	TA1535	TA98	TA1537	WP2*uvr*A
Without metabolic activation (–S9)					
0	145 ± 5	12 ± 2	21 ± 3	26 ± 7	13 ± 3
12.346	139 ± 9	14 ± 3	23 ± 3	23 ± 8	12 ± 1
37.037	152 ± 12	12 ± 3	22 ± 7	26 ± 3	14 ± 3
111.111	149 ± 12	11 ± 2	24 ± 3	26 ± 2	13 ± 2
333.333	154 ± 8	12 ± 3	24 ± 6	26 ± 4	15 ± 2
1000	155 ± 10	10 ± 4	19 ± 5	27 ± 2	18 ± 2
SA 0.5	315 ± 32	112 ± 8	-	-	-
2-NF 2	-	-	260 ± 9	-	-
9-AA 50	-	-	-	201 ± 98	-
4NQO 1	-	-	-	-	363 ± 19
2-AA 2‡	-	13 ± 3	-	-	-
BP 2‡	-	-	27 ± 10	-	-
With metabolic activation (+S9)					
0	148 ± 7	12 ± 4	30 ± 3	11 ± 1	32 ± 4
12.346	160 ± 6	9 ± 3	31 ± 4	11 ± 3	30 ± 5
37.037	159 ± 7	12 ± 3	31 ± 9	12 ± 2	29 ± 8
111.111	143 ± 16	10 ± 3	36 ± 4	12 ± 3	35 ± 5
333.333	138 ± 10	13 ± 3	36 ± 4	10 ± 3	35 ± 7
1000	149 ± 18	10 ± 3	32 ± 6	12 ± 2	31 ± 1
BP 2	644 ± 158	-	381 ± 18	95 ± 9	-
2-AA 2	-	155 ± 14	-	-	-
2-AA 4	-	-	-	-	182 ± 23

Values are mean revertant colony counts per plate (four plates per strain, concentration, and activation condition). The 0 μg/plate group is the sterile distilled water vehicle control; −S9/+S9 indicate absence/presence of metabolic activation. -, not tested for that strain. ‡ Additional 2-AA (TA1535) and BP (TA98) treatments without S9 assessed activation dependence.

PL-W, *Piper longum* hot-water extract; S9, rat liver post-mitochondrial fraction; SA, sodium azide; 2-NF, 2-nitrofluorene; 9-AA, 9-aminoacridine; 4-NQO, 4-nitroquinoline N-oxide; BP, benzo[*a*]pyrene; 2-AA, 2-aminoanthracene.

In vitro chromosomal aberration assay

The assay was performed according to OECD Test Guideline 473 [14]. Chinese hamster lung (CHL) cells obtained from the American Type Culture Collection (Manassas, VA, USA) were maintained at 37 ± 1 °C and 5% CO_2 in minimum essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells had a modal chromosome number of 25 and an approximately 15-h cell cycle and were screened for mycoplasma contamination by PCR before use. Cells were seeded at 4×10^4 cells per 25-cm^2 flask in 5.0 mL of medium and cultured for approximately three days before treatment.

PL-P and PL-W were evaluated under three conditions: 6-h treatment with S9 followed by 18-h recovery, 6-h treatment without S9 followed by 18-h recovery, and 22-h treatment without S9 followed by 2-h recovery. Concentrations were selected from preliminary concentration-range-finding experiments (Supplementary Tables S4–S7). Results for concentrations selected for metaphase scoring are presented in Tables 4 and 5. Two replicate cultures were used per concentration and treatment condition. The final S9 concentration in activated cultures was 2% (v/v). Sterile distilled water served as the vehicle control. CPA at 4.5 μg/mL served as the positive control with S9, whereas MMC at 0.1 and 0.075 μg/mL served as the positive control for the 6-h and 22-h treatments without S9, respectively.

Table 4: Chromosomal aberrations and relative population doubling in Chinese hamster lung cells treated with *Piper longum* powder (PL-P)

Concentration or control (μg/mL)	Mean aberrant metaphases Gaps included / excluded	PP	ER	RPD (%)
6-hour treatment with S9 and 18-hour recovery				
0	2.5 / 1.5	1.5	0.0	100
87.5	1.5 / 1.0	2.0	0.0	108
800	1.5 / 1.5	1.0	0.5	83
1600	1.5 / 1.5	1.5	1.0	46
CPA 4.5	10.5 / 9.5†	1.0	0.0	89
6-hour treatment without S9 and 18-hour recovery				
0	1.5 / 1.0	1.5	0.0	100
87.5	4.0 / 3.0	1.0	0.0	91
350	2.0 / 1.0	2.5	0.0	76
820	0.5 / 0.5	1.5	0.0	49
MMC 0.1	16.0 / 16.0†	1.0	0.0	70
22-hour treatment without S9 and 2-hour recovery				
0	1.5 / 1.5	1.0	0.0	100
87.5	1.0 / 1.0	1.5	0.0	88
175	0.5 / 0.5	1.0	0.0	73
350	1.0 / 1.0	2.0	0.0	45
MMC 0.075	14.0 / 12.5†	2.0	0.0	80

Aberrant metaphase values are mean counts from duplicate cultures (150 metaphases per culture), shown with gaps included/excluded. PP and ER are also mean counts. Only concentrations selected for metaphase scoring are shown. The 0 μg/mL group is the sterile distilled water vehicle control. † $p < 0.01$ versus the corresponding vehicle control (Fisher's exact test).

PL-P, *Piper longum* powder; S9, rat liver post-mitochondrial fraction; PP, polyploid metaphases; ER, endoreduplicated metaphases; RPD, relative population doubling; CPA, cyclophosphamide monohydrate; MMC, mitomycin C.

Table 5: Chromosomal aberrations and relative population doubling in Chinese hamster lung cells treated with *Piper longum* hot-water extract (PL-W).

Concentration or control (μg/mL)	Mean aberrant metaphases Gaps included / excluded	PP	ER	RPD (%)
6-hour treatment with S9 and 18-hour recovery				
0	0.5 / 0.0	1.5	0.0	100
150	3.0 / 2.0	1.0	0.5	111
500	2.0 / 1.5	2.0	0.0	114
1600	3.0 / 2.0	1.5	1.0	108
CPA 4.5	10.5 / 10.0†	2.5	0.0	101
6-hour treatment without S9 and 18-hour recovery				
0	1.0 / 1.0	1.0	0.0	100
150	2.5 / 1.5	2.5	0.0	106
500	1.0 / 1.0	2.0	0.0	103
1600	1.0 / 1.0	2.0	0.0	86
MMC 0.1	12.5 / 10.5†	2.0	0.0	89
22-hour treatment without S9 and 2-hour recovery				
0	2.5 / 2.0	2.0	0.5	100
150	3.0 / 2.0	1.0	0.0	104
500	3.0 / 1.5	1.5	0.0	82
1600	0.0 / 0.0	2.0	0.0	50
MMC 0.075	20.0 / 17.0†	1.5	0.0	93

Aberrant metaphase values are mean counts from duplicate cultures (150 metaphases per culture), shown with gaps included/excluded. PP and ER are also mean counts. Only concentrations selected for metaphase scoring are shown. The 0 μg/mL group is the sterile distilled water vehicle control. † $p < 0.01$ versus the corresponding vehicle control (Fisher's exact test).

PL-W, *Piper longum* hot-water extract; S9, rat liver post-mitochondrial fraction; PP, polyploid metaphases; ER, endoreduplicated metaphases; RPD, relative population doubling; CPA, cyclophosphamide monohydrate; MMC, mitomycin C.

Supplementary Table S3: Concentration range-finding results for *Piper longum* hot-water extract (PL-W) in the bacterial reverse mutation assay.

Concentration (μg/mL)	Visible changes	Count 1	Count 2	Mean count	PD	RPD (%)
6-hour treatment with S9						
0	–	5500	5621	5561	1.025	100
50	–	5051	5044	5048	0.886	86
100	–	5162	5090	5126	0.908	89
150	#&$∝	4716	4694	4705	0.784	76
200	#&$∝	4933	4855	4894	0.841	82
1000	#&$∝	4575	4525	4550	0.736	72
1500	#&$∝	3896	3818	3857	0.498	49
2000	#&$∝	2505	2506	2506	-0.125	-12
5000	#&$∝	1321	1322	1322	-1.048	-102
6-hour treatment without S9						
0	–	7838	7748	7793	1.512	100
50	–	7246	7280	7263	1.411	93
100	–	7121	7080	7101	1.378	91
150	#&$∝	6482	6466	6474	1.245	82
200	#&$∝	6381	6363	6372	1.222	81
1000	#&$∝	3035	3033	3034	0.151	10
1500	#&$∝	1311	1302	1307	-1.064	-70
2000	#&$∝	307	306	307	-3.156	-209
5000	#&$∝	153	150	152	-4.173	-276
22-hour treatment without S9						
0	–	7498	7338	7418	1.441	100
50	–	7063	7011	7037	1.365	95
100	–	6290	6288	6289	1.203	83
150	#&$∝	5859	5822	5841	1.096	76
200	#&$∝	5070	4924	4997	0.871	60
1000	#&$∝	1394	1399	1397	-0.968	-67
1500	#&$∝	191	201	196	-3.801	-264
2000	#&$∝	104	110	107	-4.674	-324
5000	#&$∝	359	305	332	-3.041	-211
Initial count		2726	2738	2732	–	–

One Chinese hamster lung (CHL) cell culture was used per concentration and treatment condition. Cells were exposed for 6 h with or without S9 followed by an 18-h recovery period, or for 22 h without S9 followed by a 2-h recovery period. The concentration of 0 μg/mL denotes the vehicle control (sterile distilled water).

Count 1 and Count 2 represent repeated instrument measurements from the same culture. Cell suspensions were diluted 25-fold, and a counting volume of 0.5 mL was used. Mean count is the arithmetic mean of the two measurements; total cell number per culture was calculated as mean count × 500. Initial count refers to the measurement at treatment initiation. PD was calculated as $\log_2$(cell number at harvest/cell number at treatment initiation), and RPD as 100 × PD of the treated culture/PD of the corresponding vehicle control. Negative PD and RPD values indicate that cell numbers at harvest were below those at treatment initiation. RPD represents relative cell proliferation, not percentage viability.

#, turbidity at treatment initiation; &, turbidity at treatment completion; $, precipitation at treatment initiation; ∝, precipitation at treatment completion. '–' denotes no annotation in the "Visible changes" column or a nonapplicable entry in the PD and RPD columns.

PL-P, *Piper longum* powder; CHL, Chinese hamster lung; S9, rat liver post-mitochondrial fraction used for metabolic activation; PD, population doubling; RPD, relative population doubling.

Supplementary Table S4: Cell proliferation in the first concentration range-finding experiment with *Piper longum* powder (PL-P) for the *in vitro* chromosomal aberration assay.

Concentration or control (μg/plate)	TA100	TA1535	TA98	TA1537	WP2*uvr*A
Without metabolic activation (–S9)					
0	106	12	21	8	22
8	102	8	26	8	20
40	157	14	30	9	20
200	132	10	18	8	23
1000	134	12	26	9	19
3000	92	13	19	9	18
5000	126	15	26	13	18
SA 0.5	262	144	-	-	-
2-NF 2	-	-	471	-	-
9-AA 50	-	-	-	717	-
4NQO 1	-	-	-	-	107
2-AA 2‡	-	14	-	-	-
BP 2‡	-	-	21	-	-
With metabolic activation (+S9)					
0	121	15	39	9	26
8	116	13	31	8	25
40	118	14	31	9	26
200	123	12	33	9	28
1000	110	10	43	10	27
3000	132	13	43	12	30
5000	126	12	36	7	31
BP 2	885	-	382	73	-
2-AA 2	-	154	-	-	-
2-AA 4	-	-	-	-	114

Values represent revertant colony counts from one plate per strain, concentration, and metabolic activation condition. TA100, TA1535, TA98, and TA1537 are *Salmonella typhimurium* strains; WP2*uvr*A is an *Escherichia coli* strain. The concentration of 0 μg/plate denotes the vehicle control (sterile distilled water). Numbers accompanying control compounds indicate doses in μg/plate. –S9 and +S9 indicate the absence and presence of metabolic activation, respectively. '-' indicates that the control was not used for the corresponding strain.

‡ Additional treatments with 2-AA in TA1535 and BP in TA98 were conducted without S9 to assess the dependence of their responses on metabolic activation.

Precipitation was observed at ≥1000 μg/plate upon mixing with top agar and at ≥200 μg/plate after 48 h of incubation. Turbidity was observed at ≥3000 μg/plate upon mixing. These observations applied to all strains with and without S9. No bacterial toxicity was observed.

PL-P, *Piper longum* powder; S9, rat liver post-mitochondrial fraction used for metabolic activation; SA, sodium azide; 2-NF, 2-nitrofluorene; 9-AA, 9-aminoacridine; 4NQO, 4-nitroquinoline N-oxide; BP, benzo[*a*]pyrene; 2-AA, 2-aminoanthracene.

Supplementary Table S5: Cell proliferation in the second concentration range-finding experiment with *Piper longum* powder (PL-P) for the *in vitro* chromosomal aberration assay.

Concentration (µg/mL)	Visible changes	Count 1	Count 2	Mean count	PD	RPD (%)
6-hour treatment with S9						
0	–	7750	7677	7714	1.427	100
800	#&$∝	6537	6549	6543	1.190	83
900	#&$∝	6116	6196	6156	1.102	77
1000	#&$∝	5581	5619	5600	0.965	68
1100	#&$∝	5158	5140	5149	0.844	59
1500	#&$∝	4459	4248	4354	0.602	42
1600	#&$∝	4590	4565	4578	0.675	47
6-hour treatment without S9						
0	–	8854	8750	8802	1.618	100
300	#&$∝	7159	7072	7116	1.311	81
400	#&$∝	6104	6182	6143	1.099	68
500	#&$∝	5894	5798	5846	1.027	64
600	#&$∝	5779	5827	5803	1.017	63
700	#&$∝	5587	5626	5607	0.967	60
800	#&$∝	5059	5032	5046	0.815	50
1000	#&$∝	4261	4170	4216	0.556	34
22-hour treatment without S9						
0	–	8171	8235	8203	1.516	100
200	#&$∝	5686	5626	5656	0.980	65
250	#&$∝	5672	5613	5643	0.976	64
300	#&$∝	5003	5036	5020	0.807	53
400	#&$∝	4801	4741	4771	0.734	48
500	#&$∝	4238	4411	4325	0.592	39
600	#&$∝	3822	3680	3751	0.387	26
700	#&$∝	3140	3100	3120	0.122	8
Initial count		2903	2833	2868	–	–

One Chinese hamster lung (CHL) cell culture was used per concentration and treatment condition. Cells were exposed for 6 h with or without S9 followed by an 18-h recovery period, or for 22 h without S9 followed by a 2-h recovery period. The concentration of 0 µg/mL denotes the vehicle control (sterile distilled water).

Count 1 and Count 2 represent repeated instrument measurements from the same culture. Cell suspensions were diluted 25-fold, and a counting volume of 0.5 mL was used. Mean count is the arithmetic mean of the two measurements; total cell number per culture was calculated as mean count × 500. Initial count refers to the measurement at treatment initiation. PD was calculated as $\log_2$(cell number at harvest/cell number at treatment initiation), and RPD as 100 × PD of the treated culture/PD of the corresponding vehicle control. RPD represents relative cell proliferation, not percentage viability.

#, turbidity at treatment initiation; &, turbidity at treatment completion; $, precipitation at treatment initiation; ∝, precipitation at treatment completion. '–' denotes no annotation in the "Visible changes" column or a nonapplicable entry in the PD and RPD columns.

PL-P, *Piper longum* powder; CHL, Chinese hamster lung; S9, rat liver post-mitochondrial fraction used for metabolic activation; PD, population doubling; RPD, relative population doubling.

Supplementary Table S6: Cell proliferation in the first concentration range-finding experiment with *Piper longum* hot-water extract (PL-W) for the *in vitro* chromosomal aberration assay.

Concentration (μg/mL)	Visible changes	Count 1	Count 2	Mean count	PD	RPD (%)
6-hour treatment with S9						
0	–	7115	7007	7061	1.237	100
50	–	7471	7343	7407	1.306	106
100	–	8188	8022	8105	1.436	116
150	–	7968	7779	7874	1.394	113
200	–	8316	8192	8254	1.462	118
1000	#&$∝	8709	8640	8675	1.534	124
1500	#&$∝	7639	7581	7610	1.345	109
2000	#&$∝	6852	6746	6799	1.182	96
5000	#&$∝	6031	5908	5970	0.995	80
6-hour treatment without S9						
0	–	9478	9379	9429	1.654	100
50	–	9748	9720	9734	1.700	103
100	–	9167	9108	9138	1.609	97
150	–	9224	9055	9140	1.609	97
200	–	9166	9004	9085	1.600	97
1000	#&$∝	7513	7431	7472	1.318	80
1500	#&$∝	7930	7875	7903	1.399	85
2000	#&$∝	7935	7825	7880	1.395	84
5000	#&$∝	5279	5244	5262	0.812	49
22-hour treatment without S9						
0	–	10057	10071	10064	1.748	100
50	–	9241	9272	9257	1.627	93
100	–	8941	9017	8979	1.584	91
150	–	8984	9009	8997	1.586	91
200	–	8758	8787	8773	1.550	89
1000	#&$∝	6408	6316	6362	1.086	62
1500	#&$∝	5442	5409	5426	0.857	49
2000	#&$∝	5256	5124	5190	0.793	45
5000	#&$∝	2260	2290	2275	-0.397	-23
Initial count		2949	3043	2996	–	–

One Chinese hamster lung (CHL) cell culture was used per concentration and treatment condition. Cells were exposed for 6 h with or without S9 followed by an 18-h recovery period, or for 22 h without S9 followed by a 2-h recovery period. The concentration of 0 μg/mL denotes the vehicle control (sterile distilled water).

Count 1 and Count 2 represent repeated instrument measurements from the same culture. Cell suspensions were diluted 25-fold, and a counting volume of 0.5 mL was used. Mean count is the arithmetic mean of the two measurements; total cell number per culture was calculated as mean count × 500. Initial count refers to the measurement at treatment initiation. PD was calculated as $\log_2$(cell number at harvest/cell number at treatment initiation), and RPD as 100 × PD of the treated culture/PD of the corresponding vehicle control. RPD represents relative cell proliferation, not percentage viability. Values above 100% indicate greater population doubling than in the corresponding vehicle control. Negative PD and RPD values indicate that cell numbers at harvest were below those at treatment initiation.

#, turbidity at treatment initiation; &, turbidity at treatment completion; $, precipitation at treatment initiation; ∝, precipitation at treatment completion. '–' denotes no annotation in the "Visible changes" column or a nonapplicable entry in the PD and RPD columns.

PL-W, *Piper longum* hot-water extract; CHL, Chinese hamster lung; S9, rat liver post-mitochondrial fraction used for metabolic activation; PD, population doubling; RPD, relative population doubling.

Supplementary Table S7: Cell proliferation in the second concentration range-finding experiment with *Piper longum* hot-water extract (PL-W) for the *in vitro* chromosomal aberration assay.

Concentration (μg/mL)	Visible changes	Count 1	Count 2	Mean count	PD	RPD (%)
22-hour treatment without S9						
0	–	10719	10803	10761	1.793	100
800	#&	7045	6938	6992	1.171	65
1000	#&$∝	6680	6572	6626	1.094	61
1200	#&$∝	6542	6605	6574	1.082	60
1400	#&$∝	6004	5939	5972	0.944	53
1600	#&$∝	5758	5858	5808	0.904	50
1800	#&$∝	5425	5340	5383	0.794	44
2000	#&$∝	4064	4138	4101	0.402	22
Initial count		3143	3066	3105	–	–

One Chinese hamster lung (CHL) cell culture was used per concentration. Cells were exposed for 22 h without S9 followed by a 2-h recovery period. The concentration of 0 μg/mL denotes the vehicle control (sterile distilled water).

Count 1 and Count 2 represent repeated instrument measurements from the same culture. Cell suspensions were diluted 25-fold, and a counting volume of 0.5 mL was used. Mean count is the arithmetic mean of the two measurements; total cell number per culture was calculated as mean count × 500. Initial count refers to the measurement at treatment initiation. PD was calculated as $\log_2$(cell number at harvest/cell number at treatment initiation), and RPD as 100 × PD of the treated culture/PD of the corresponding vehicle control. RPD represents relative cell proliferation, not percentage viability.

#, turbidity at treatment initiation; &, turbidity at treatment completion; $, precipitation at treatment initiation; ∝, precipitation at treatment completion. '–' denotes no annotation in the "Visible changes" column or a nonapplicable entry in the PD and RPD columns.

PL-W, *Piper longum* hot-water extract; CHL, Chinese hamster lung; S9, rat liver post-mitochondrial fraction used for metabolic activation; PD, population doubling; RPD, relative population doubling.

Precipitation and turbidity were assessed visually at the beginning and end of treatment. During preliminary range-finding experiments, medium pH and osmolality were measured in the vehicle control and the highest-concentration group (5000 μg/mL) at both time points. At the end of treatment, cultures were washed once with calcium- and magnesium-free Dulbecco's phosphate-buffered saline and incubated in fresh medium for the designated recovery period. Colchicine was added at a final concentration of 1 μM approximately 22 h after treatment initiation, and cells were harvested 2 h later. Cell numbers were determined using an automated cell counter. Population doubling (PD) was calculated as $\log_2$(N final/N initial), where N initial and N final denote cell numbers at treatment initiation and harvest, respectively. Relative population doubling (RPD) was calculated as 100 × PD treated/PD vehicle control. Cells were treated with 75 mM potassium chloride for 10 min at room temperature and fixed with methanol and glacial acetic acid (3:1, v/v). Two slides were prepared per culture and stained with 3% (v/v) Giemsa solution in Gurr buffer for 7 min. One suitable slide per culture was selected and coded before microscopic evaluation. For structural aberration assessment, 150 well-spread metaphases containing 23–27 chromosomes were examined per culture at ×1000 magnification. Chromatid- and chromosome-type gaps, breaks, exchanges, and multiple aberrations were recorded. Gaps were recorded separately, and aberrant metaphases were tabulated both including and excluding gaps. An additional 150 metaphases per culture were examined for polyploidy (PP; ≥37 centromeres) and endoreduplication (ER), irrespective of the presence of structural aberrations.

Mouse bone marrow micronucleus assay

The mouse bone marrow micronucleus assay was conducted according to OECD Test Guideline 474 [15]. PL-P and PL-W were evaluated in separate experiments. For each preparation, six mice were allocated to each of five groups: a vehicle control, three treatment groups receiving 500, 1000, or 2000 mg/kg, and a positive-control group.

The test articles or vehicle (sterile distilled water) were administered orally gavage at 20 mL/kg once daily for two consecutive days. CPA, prepared in sterile saline, was administered to the positive-control group as a single intraperitoneal dose of 70 mg/kg at 10 mL/kg at the time of the second test-article administration. Clinical signs and body weights were monitored in all six mice per group through Day 3 (Supplementary Table S8 and S9). Body weights were recorded before dosing on Days 1 and 2 and on Day 3. The stability, homogeneity, and concentration of the dosing formulations were not analytically verified.

Supplementary Table S8: Individual body weights and clinical observations in male ICR mice administered *Piper longum* powder (PL-P) in the bone marrow micronucleus assay

Group	Dose (mg/kg)	Animal no.	Day 1 body weight (g)	Day 2 body weight (g)	Day 3 body weight (g)	Clinical observation
Vehicle control	0	1	34.2	34.8	34.1	No abnormalities detected
		2	34.6	35.3	34.7	No abnormalities detected
		3	31.7	32.4	31.9	No abnormalities detected
		4	33.5	33.5	33.7	No abnormalities detected
		5	34.1	34.5	34.5	No abnormalities detected
		6	35.5	36.0	36.1	No abnormalities detected
PL-P	500	7	34.2	34.5	35.2	No abnormalities detected
		8	32.7	33.1	33.1	No abnormalities detected
		9	35.6	36.2	36.6	No abnormalities detected
		10	35.7	36.3	36.8	No abnormalities detected
		11	33.7	34.3	34.0	No abnormalities detected
		12	32.3	32.1	32.7	No abnormalities detected
	1000	13	32.8	33.0	33.0	No abnormalities detected
		14	33.2	33.5	33.2	No abnormalities detected
		15	33.5	33.7	34.3	No abnormalities detected
		16	34.3	34.7	35.6	No abnormalities detected
		17	34.9	35.3	35.0	No abnormalities detected
		18	34.8	35.3	35.5	No abnormalities detected
	2000	19	35.5	35.5	34.2	No abnormalities detected
		20	34.0	34.4	33.8	No abnormalities detected
		21	34.8	34.0	33.6	No abnormalities detected
		22	32.6	31.4	31.7	No abnormalities detected
		23	33.7	32.7	33.4	No abnormalities detected
		24	32.4	32.1	32.0	No abnormalities detected
Positive control	70	25	36.5	37.3	38.0	No abnormalities detected
		26	34.2	34.9	34.1	No abnormalities detected
		27	34.2	35.3	35.3	No abnormalities detected
		28	33.8	34.6	34.0	No abnormalities detected
		29	33.1	34.2	33.8	No abnormalities detected
		30	32.6	33.3	33.4	No abnormalities detected

Body weights are individual observations from six animals assigned to each group. The vehicle-control and PL-P groups received sterile distilled water or PL-P orally at 20 mL/kg once daily on study days 1 and 2. The positive-control group received CPA once intraperitoneally at 70 mg/kg on study day 2. Clinical signs were monitored during the three-day observation period; no abnormal clinical signs were detected, and all animals survived until scheduled sacrifice on day 3. Although six animals were evaluated for body weight and clinical signs in each group, five animals per group were used for micronucleus scoring.

CPA, cyclophosphamide monohydrate; PL-P, *Piper longum* powder.

Supplementary Table S9: Individual body weights and clinical observations in male ICR mice administered *Piper longum* hot-water extract (PL-W) in the bone marrow micronucleus assay

Group	Dose (mg/kg)	Animal no.	Day 1 body weight (g)	Day 2 body weight (g)	Day 3 body weight (g)	Clinical observation
Vehicle control	0	1	32.5	32.7	33.0	No abnormalities detected
		2	33.8	33.5	33.7	No abnormalities detected
		3	36.1	36.4	36.6	No abnormalities detected
		4	33.7	33.9	34.3	No abnormalities detected
		5	35.5	35.7	36.2	No abnormalities detected
		6	35.4	35.0	35.0	No abnormalities detected
PL-W	500	7	35.2	36.1	36.4	No abnormalities detected
		8	33.6	33.8	33.6	No abnormalities detected
		9	34.1	33.9	34.2	No abnormalities detected
		10	33.5	33.9	33.9	No abnormalities detected
		11	32.3	32.3	32.4	No abnormalities detected
		12	34.7	34.7	34.7	No abnormalities detected
	1000	13	33.6	33.5	34.0	No abnormalities detected
		14	33.9	34.1	33.9	No abnormalities detected
		15	35.5	36.1	36.8	No abnormalities detected
		16	34.5	35.5	35.4	No abnormalities detected
		17	33.1	33.6	34.3	No abnormalities detected
		18	31.6	31.2	31.0	No abnormalities detected
	2000	19	33.5	33.7	34.3	No abnormalities detected
		20	30.7	31.4	31.9	No abnormalities detected
		21	35.1	35.5	35.7	No abnormalities detected
		22	33.6	34.1	33.5	No abnormalities detected
		23	34.1	35.2	35.5	No abnormalities detected
		24	36.0	36.6	37.2	No abnormalities detected
Positive control	70	25	32.2	32.7	33.1	No abnormalities detected
		26	33.2	33.6	33.0	No abnormalities detected
		27	34.6	36.0	35.9	No abnormalities detected
		28	36.3	36.8	35.9	No abnormalities detected
		29	32.8	33.7	32.7	No abnormalities detected
		30	33.7	33.9	33.8	No abnormalities detected

Body weights are individual observations from six animals assigned to each group. The vehicle-control and PL-W groups received sterile distilled water or PL-W orally at 20 mL/kg once daily on study days 1 and 2. The positive-control group received CPA once intraperitoneally at 70 mg/kg on study day 2. Clinical signs were monitored during the three-day observation period; no abnormal clinical signs were detected, and all animals survived until scheduled sacrifice on day 3. Although six animals were evaluated for body weight and clinical signs in each group, five animals per group were used for micronucleus scoring.

CPA, cyclophosphamide monohydrate; PL-W, *Piper longum* hot-water extract.

Approximately 23 h after the final administration, mice were euthanized with CO_2, and femoral bone marrow was collected. Bone marrow was flushed with 2 mL of fetal bovine serum and filtered through an α-cellulose/Sigmacell cellulose column (1:1, v/v). After the addition of 1 mL of Hanks' balanced salt solution, the suspension was centrifuged at 1000 rpm for 5 min at room temperature. The supernatant was removed, and two smears were prepared per animal from the remaining cell suspension. Smears were air-dried, fixed in methanol for 5 min, and stained with 5% (v/v) Giemsa solution prepared in Gurr buffer.

The first five animals in animal-number order within each group were evaluated for micronuclei. One suitably stained smear per animal was selected and randomly coded before microscopic evaluation. For each animal, micronucleated polychromatic erythrocytes (MNPCEs) were counted among 4000 polychromatic erythrocytes (PCEs). The endpoint was expressed as the number of MNPCEs per 4000 PCEs. In addition, 500 erythrocytes per animal were classified as PCEs or normochromatic erythrocytes (NCEs), irrespective of micronucleus status. The proportion of immature erythrocytes, calculated as PCE/(PCE + NCE), was used as an indicator of bone marrow cytotoxicity.

Statistical Analysis

For the bacterial reverse mutation assay, no inferential statistical tests were performed. Mean revertant colony counts were compared descriptively with the corresponding vehicle controls, with interpretation based on the magnitude and concentration dependence of the responses and their biological relevance.

For the *in vitro* chromosomal aberration assay, aberrant metaphases including and excluding gaps and the combined polyploid and endoreduplicated metaphases endpoint (PP + ER) were analyzed using SAS. Test-article groups were initially compared with the vehicle control using a chi-square test. When $p < 0.05$, Fisher's exact test and the Cochran–Armitage trend test were applied to assess group differences and concentration-related trends, respectively. Positive-control comparisons used Fisher's exact test. Interpretation of structural chromosomal damage emphasized aberrations excluding gaps.

For the mouse bone marrow micronucleus assay, MNPCE counts were summarized as means ± standard deviations for five mice per group. Test-article groups were compared with the concurrent vehicle control using the Kruskal–Wallis test and Dunn's rank-sum test. The protocol specified a Cochran–Armitage trend test following a significant result; this test was not performed because the initial comparisons yielded $p \geq 0.05$. Positive-control MNPCE counts were compared with the vehicle control using the Mann–Whitney U-test. PCE/(PCE + NCE) ratios were arcsine square-root transformed before analysis. Test-article comparisons used one-way analysis of variance, followed by Dunnett's test when significant; positive-control comparisons used Student's t-test. The individual mouse was the experimental unit, and cells scored within an animal were subsamples.

Body weights were analyzed for six mice per group. Homogeneity of variances was assessed using Bartlett's test. Homogeneous data were evaluated using one-way analysis of variance followed by Dunnett's test; heterogeneous data were evaluated using the Kruskal–Wallis test followed by Dunn's rank-sum test. *In vivo* analyses used Pristima (version 7.4) and/or SAS/STAT (version 9.4). A p value < 0.05 was considered statistically significant for inferential analyses. Historical control data were considered separately when evaluating assay performance.

Results and Discussion

Chemical differences between the preparations

HPLC analysis revealed differences in the contents of the four measured marker compounds between PL-P and PL-W (Table 1). Piperine was the most abundant of these markers in both preparations, with contents of 2.427% in PL-P and 0.414% in PL-W. The corresponding contents of pellitorine, pipernonaline, and guineensine were 0.138%, 0.301%, and 0.115% in PL-P and 0.008%, 0.021%, and 0.008% in PL-W, respectively. Thus, PL-W contained lower concentrations of all four markers than PL-P.

These compositional differences support evaluating the two preparations separately, because equal doses of PL-P and PL-W do not contain equivalent amounts of the measured constituents. However, marker concentrations in the finished preparations do not establish extraction recoveries relative to the starting herbal material or distinguish incomplete extraction from chemical transformation during processing. Furthermore, the four-marker analysis characterizes only part of the chemical composition of these preparations. The genotoxicity findings therefore apply to the tested preparations as mixtures and cannot be attributed to individual markers solely on the basis of their concentrations. Quality testing of the source herbal material showed no detectable residues of the pesticides examined and a sulfur dioxide content of 3 ppm. Loss on drying and ash content were 8.7% and 4.6%, respectively (Supplementary Table S1).

Bacterial reverse mutation findings

Neither PL-P nor PL-W produced a biologically relevant increase in revertant colony counts in *Salmonella typhimurium* TA100, TA1535, TA98, and TA1537 or *Escherichia coli* WP2*uvr*A over the main-study concentration range of 12.346–1000 µg/plate, with or without S9 metabolic activation (Tables 2 and 3). Colony counts generally remained close to the corresponding vehicle-control values. Small numerical increases occurred in some treatment groups; for example, WP2*uvr*A counts without S9 increased from 13 colonies/plate in the vehicle controls to 18 colonies/plate at 1000 µg/plate for both preparations. Considered together with the concentration profiles and preliminary findings, these changes did not indicate a biologically relevant mutagenic response.

The strain-specific positive controls increased revertant colony counts in both activation conditions. In addition, 2-aminoanthracene in TA1535 and benzo[*a*]pyrene in TA98 produced pronounced responses with S9, whereas their responses without S9 remained close to the corresponding vehicle-control values. These findings supported the responsiveness of the bacterial strains and the functional activity of the metabolic activation system.

No background-lawn thinning, microcolony formation, or reduction of at least 50% in revertant colony counts was observed with either preparation. Precipitation nevertheless occurred during the main experiment at PL-P concentrations of 333.333 μg/plate and above and at 1000 μg/plate for PL-W, irrespective of S9 activation. The preliminary experiments extended to 5000 μg/plate and likewise showed no clear mutagenic response or overt bacterial toxicity (Supplementary Tables S2 and S3). However, these experiments used one plate per concentration and condition, whereas the main experiment used four plates. Consequently, the replicated evidence supporting the negative findings extends to 1000 μg/plate.

The results support the absence of detectable bacterial mutagenic activity for both preparations under the conditions examined. OECD TG 471 identifies solubility and cytotoxicity as relevant considerations in concentration selection and emphasizes biological relevance in interpreting colony-count changes [13]. Here, precipitation indicates that nominal preparation concentrations cannot be assumed to represent fully dissolved exposure. The negative findings are also consistent with the negative bacterial mutagenicity result for piperine reported by the National Toxicology Program [17]. However, this constituent-level evidence does not establish the contributions of piperine or other compounds to the responses of the whole preparations. Because bacterial mutation assays do not reproduce all aspects of mammalian uptake and metabolism, these findings should be considered together with the chromosomal aberration and micronucleus results.

Chromosomal aberrations in CHL cells

Neither PL-P nor PL-W significantly increased the frequency of metaphases with structural chromosomal aberrations excluding gaps under any of the three treatment conditions (Tables 4 and 5). Mean numbers of aberrant metaphases excluding gaps ranged from 0.5 to 3.0 per culture for PL-P and from 0.0 to 2.0 per culture for PL-W, with 150 metaphases examined per culture. No consistent concentration-related increase was evident. The combined frequency of polyploid and endoreduplicated metaphases (PP + ER) also showed no statistically significant increase. In comparison, CPA and MMC increased structural chromosomal aberrations in the corresponding treatment conditions ($p < 0.01$), demonstrating the responsiveness of the assay.

The preparations differed in their effects on cell proliferation. For PL-P, the highest concentrations evaluated for chromosomal aberrations were 1600 μg/mL after 6-h treatment with S9, 820 μg/mL after 6-h treatment without S9, and 350 μg/mL after 22-h treatment without S9. The corresponding RPD values were 46%, 49%, and 45%. For PL-W, the highest evaluated concentration was 1600 μg/mL under all three conditions, with RPD values of 108%, 86%, and 50%, respectively. Thus, PL-P inhibited proliferation under all three conditions, whereas pronounced inhibition by PL-W occurred after prolonged exposure without S9. The RPD values of 45–50% were within the range corresponding to the cytotoxicity target recommended by OECD TG 473 when cytotoxicity determines the highest concentration [14]. In these cultures, substantial inhibition of proliferation was not accompanied by an increase in structural chromosomal damage.

Visible turbidity and precipitation occurred at all evaluated PL-P concentrations at the beginning and end of treatment. For PL-W, precipitation was observed initially at concentrations of 500 μg/mL and above, while both turbidity and precipitation were present at 1600 μg/mL at the beginning and end of treatment. Preliminary testing at 5000 μg/mL showed no vehicle-relative changes of ≥1 pH unit or ≥50 mOsm/kg in osmolality. Nevertheless, precipitation introduces uncertainty regarding the dissolved concentrations available to cells. The observed inhibition of proliferation demonstrates a cellular response to treatment but does not establish intracellular exposure to each constituent.

Published studies of individual Piper constituents provide context for these findings. Piperine was negative in an *in vitro* micronucleus assay in Chinese hamster ovary cells [9], whereas piplartine (piperlongumine) induced chromosomal aberrations and micronuclei in V79 cells [18]. These studies involved isolated compounds and different cellular systems, and their outcomes cannot be directly extrapolated to PL-P or PL-W. Piplartine was also not among the four markers quantified in the present study. The present findings therefore support the absence of detectable clastogenic activity of the tested preparations in CHL cells under the conditions examined. The absence of an increase in PP + ER provides supplementary information on ploidy changes but does not exclude aneugenic activity, which is not comprehensively assessed by this assay [14].

Bone marrow micronuclei in mice

Oral administration of PL-P or PL-W at 500, 1000, and 2000 mg/kg for two consecutive days did not significantly increase bone marrow MNPCE counts relative to the corresponding vehicle controls (Table 6). Mean counts for PL-P were 6.40, 6.80, and 6.00 MNPCEs per 4000 PCEs, respectively, compared with 6.00 in the vehicle control. Corresponding values for PL-W were 5.60, 5.00, and 5.00, compared with 5.00 in its vehicle control. Neither preparation showed a consistent dose-related pattern of increasing MNPCE counts.

Table 6: Bone marrow micronucleus assay results in male ICR mice administered *Piper longum* powder (PL-P) or hot-water extract (PL-W).

Group	Dose (mg/kg)	MNPCEs per 4000 PCEs	PCE / (PCE + NCE)
PL-P study			
VC	0	6.00 ± 1.87	0.43 ± 0.06
PL-P	500	6.40 ± 2.79	0.42 ± 0.08
	1000	6.80 ± 1.64	0.48 ± 0.07
	2000	6.00 ± 1.22	0.38 ± 0.04
CPA	70	110.40 ± 22.47†	0.44 ± 0.07
PL-W study			
VC	0	5.00 ± 1.22	0.50 ± 0.09
PL-W	500	5.60 ± 1.82	0.48 ± 0.10
	1000	5.00 ± 1.58	0.43 ± 0.07
	2000	5.00 ± 1.22	0.43 ± 0.07
CPA	70	108.60 ± 13.28†	0.37 ± 0.04

Values are mean ± standard deviation (n = 5 mice per group). MNPCEs are counts per 4000 PCEs; the PCE/(PCE + NCE) ratio was determined from 500 erythrocytes per animal. The 0 mg/kg groups received sterile distilled water. † $p < 0.01$ versus the corresponding vehicle control (Mann–Whitney U-test).

PL-P, *Piper longum* powder; PL-W, *Piper longum* hot-water extract; VC, vehicle control; CPA, cyclophosphamide monohydrate; MNPCE, micronucleated polychromatic erythrocyte; PCE, polychromatic erythrocyte; NCE, normochromatic erythrocyte.

The proportion of immature erythrocytes, expressed as PCE/(PCE + NCE), did not differ significantly between the treatment groups and their respective vehicle controls. Mean proportions ranged from 0.38 to 0.48 for PL-P and from 0.43 to 0.48 for PL-W, compared with vehicle-control values of 0.43 and 0.50, respectively. No deaths or abnormal clinical signs were observed in the test-article groups, and body weights did not differ significantly from those of the vehicle controls. Individual body weights and clinical observations are provided in Supplementary Tables S8 and S9.

CPA significantly increased MNPCE counts in both experiments ($p < 0.01$), with means of 110.40 ± 22.47 for PL-P and 108.60 ± 13.28 for PL-W, compared with vehicle-control means of 6.00 ± 1.87 and 5.00 ± 1.22 per 4000 PCEs, respectively. Both concurrent vehicle-control means were within the laboratory's historical male vehicle-control range of 2–12 MNPCEs per 4000 PCEs (2015–2019; data not shown). These observations support the responsiveness of the micronucleus assay.

The absence of increased MNPCE counts is consistent with the findings of Thiel *et al*., who observed no increase in bone marrow micronuclei in mice treated with isolated piperine up to the maximum tolerated dose [9]. However, constituent-level findings cannot establish the *in vivo* genotoxicity profile of whole herbal preparations, whose composition and disposition may differ from those of an isolated compound.

A further limitation is that bone marrow exposure was not established for either preparation. The absence of a significant reduction in the immature erythrocyte proportion provides no evidence of marrow toxicity through this endpoint, but it does not demonstrate adequate exposure of erythroid precursor cells. OECD TG 474 and EFSA guidance identify evidence of target-tissue exposure as an important consideration when interpreting a negative micronucleus result [15, 19]. Accordingly, the present observations support the absence of detectable micronucleus induction in the sampled bone marrow under the dosing conditions examined. They do not provide a definitive exclusion of *in vivo* genotoxicity, given the unestablished target-tissue exposure and uncertainty in the PL-P positive-control response.

Implications and limitations

The combined chemical characterization and genotoxicity findings provide complementary evidence for assessing PL-P and PL-W. Neither preparation produced a biologically relevant bacterial reverse mutation response or a statistically significant increase in structural chromosomal aberrations in CHL cells under the conditions examined. The differences in marker contents and effects on cell proliferation support interpreting these findings in relation to each preparation. Lower marker concentrations in PL-W do not, by themselves, establish lower genotoxic potential.

Precipitation during the *in vitro* experiments introduces uncertainty regarding dissolved concentrations, and nominal concentrations do not establish intracellular exposure to individual constituents. Chemical characterization was restricted to four markers and did not comprehensively describe the mixtures or identify the constituents responsible for inhibition of cell proliferation. In the micronucleus experiments, dosing formulations were not analyzed for stability, homogeneity, or concentration, adding uncertainty to characterization of the administered preparations.

The *in vivo* experiments showed no statistically significant increase in bone marrow micronuclei, but target-tissue exposure was not established. Unchanged immature erythrocyte proportions cannot independently confirm adequate marrow exposure [15, 19]. The use of male mice also limits direct assessment of responses in females. These limitations qualify the biological scope of the

findings despite the significant responses in both positive-control groups.

These findings apply to the tested preparations and experimental conditions. Their generalizability to other *P. longum* products depends on comparability of botanical material, processing, and chemical composition. The study does not establish safety during long-term human use, exclude non-genotoxic mechanisms of carcinogenesis, or define a clinical dose or quantitative human safety margin. Additional evidence concerning systemic and bone marrow exposure to relevant constituents or metabolites would strengthen interpretation of the *in vivo* findings.

Conclusion

Neither PL-P nor PL-W produced a biologically relevant bacterial mutagenic response or a statistically significant increase in structural chromosomal aberrations in CHL cells. No statistically significant increase in mouse bone marrow micronuclei was detected. These findings provide preparation-specific evidence for the genotoxicity assessment of *P. longum* under the conditions tested. Interpretation remains limited by precipitation *in vitro* and the absence of demonstrated bone marrow exposure *in vivo*. The results do not establish safety for long-term human use.

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Conflict of Interest

The authors declare no competing interests.

Data Availability

The data supporting the findings of this study are included in this article and its supplementary materials. Additional datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

N.-Y.K.: Writing–original draft, Formal analysis, Data curation. S.-M.B.: Writing–original draft, Investigation, Formal analysis, Conceptualization, Methodology. D.Y.K.: Writing–original draft, Investigation, Formal analysis, Conceptualization, Methodology. N.-Y.J.: Investigation, Data curation. K.-H.H: Investigation, review & editing Y.-B.K.: Investigation, review & editing. S.-H.P.: Writing–original draft, review & editing, Supervision, Investigation, Conceptualization, Funding acquisition.

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