Reduced genotoxicity of lignin-derivable replacements to bisphenol A studied using *in silico, in vitro*, and *in vivo* methods

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Abstract

Bisguaiacols, lignin-derivable bisphenols, are considered promising and possibly safer alternatives to bisphenol A (BPA), but comprehensive toxicity investigations are needed to ensure safety. Most toxicity studies of BPA and its analogues have focused on potential estrogenic activity, and only limited toxicological data are available on other toxicity aspects, such as genotoxicity at low exposure levels. In this study, the genotoxicity of six lignin-derivable bisguaiacols with varying regioisomer contents and degrees of methoxy substitution was investigated using a multi-tiered method, consisting of *in silico* simulations, *in vitro* Ames tests, and *in vivo* comet tests. The toxicity estimation software tool, an application that predicts toxicity of chemicals using quantitative structure-activity relationships, calculated that the majority of the lignin-derivable bisguaiacols were non-mutagenic. These results were supported by Ames tests using five tester strains (TA98, TA100, TA102, TA1535, and TA1537) at concentrations ranging from 0.5 pmol/plate to 5 nmol/plate. The potential genotoxicity of bisguaiacols was further evaluated using *in vivo* comet testing in fetal chicken livers, and in addition to the standard alkaline comet assay, the formamidopyrimidine DNA glycosylase enzyme-modified comet assay was employed to investigate oxidative DNA damage in the liver samples. Theoxidative stress analyses indicated that the majority of lignin-derivable analogues showed no signs of mutagenicity (mutagenic index < 1.5) or genotoxicity, in comparison to BPA and bisphenol F, likely due to the methoxy groups on the lignin-derivable aromatics. These findings reinforce the potential of lignin-derivable bisphenols as safer alternatives to BPA.

Keywords

Bisguaiacols; Lignin-derivable; Genotoxicity; Ames test; DNA damage; Comet assay

Abbreviations

ANOVA, analysis of variance; BGM, bisguaiacol M; BGP, bisguaiacol P; BGS, bisguaiacol S; BPA, bisphenol A; BPAF, bisphenol AF; BPF, bisphenol F; BPS, bisphenol S; BSA, bovine serum albumin; BW, body weight; DI, deionized; DMSO, dimethylsulfoxide; EA, estrogenic activity; EDTA, ethylenediamine tetraacetic acid; EMS, ethyl methanesulfonate; EW, egg weight; Fpg, formamidopyrimidine DNA glycosylase; HBSS, Hank's balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MDA, malondialdehyde; MI, mutagenic index;

MMC, mitomycin C; MW, molecular weight; OECD, Organization for Economic Co-operation and Development; PBS, phosphate-buffered saline; QSAR, quantitative structure-activity relationship; ROS, reactive oxygen species; S9 mix, metabolic activation mixture S9; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substance; T.E.S.T., toxicity estimation software tool; UD, University of Delaware; VC, vehicle control; 2AF, 2-aminofluorene; 2NF, 2-nitrofluorene

1. Introduction

Bisphenol A (BPA) is an important building block in an array of polymeric systems, such as polycarbonates, polysulfones, epoxy resins, *etc.*, [1, 2, 3], and global demand for BPA is increasing [4]. BPA-based polymers are major constituents in food contact materials due to their chemical inertness, hydrolytic stability, mechanical strength, and high-temperature stability [1, 2, 3, 5]; however, BPA can potentially leach out from these polymeric products into foodstuffs and be ingested by humans [6]. In addition to food contact materials, BPA can be present in other consumer and industrial products, such as thermal paper and medical materials [7, 8]. These broad applications allow BPA to distribute across the environment in water, sediment, food, humans, and other animals [9]. Related to this distribution, various toxicological problems related to BPA exposure have been reported, including endocrine disruption, genotoxicity, and developmental toxicity [10]. Because of these toxicity concerns, BPA usage has been restricted in various infant-related products and thermal paper in the United States, Canada, and the European Union [4, 11].

To address the adverse health effects surrounding BPA exposure, the use of commercial (petroleum-derived) BPA analogues, such as bisphenol F (BPF), bisphenol S (BPS), bisphenol AF (BPAF), *etc*., has been increasing. However, these bisphenolic replacements have similar chemical structures to BPA, which has led to concerns about their toxicological profiles, such as their endocrine disruption ability [12, 13, 14]. For example, the estrogenic activity (EA) and anti-androgenic activity of BPF, BPAF, and BPS have been demonstrated in different cell lines [13, 14]. Potential genotoxicity is also a concern, as it has been reported that two BPA analogues (bisphenol B and BPF) can induce reactive oxygen species (ROS) overproduction, lipid peroxidation, and DNA damage in human peripheral blood cells [15]. Furthermore, BPF and BPAF exert higher DNA double-strand breaks than BPA in human HepG2 cells when assessed by

a γ-H2AX assay [16]. Taken together, these toxicology concerns regarding petroleum-derived BPA alternatives provide motivation for the investigation of less toxic, sustainable alternatives to BPA and its commercial analogues.

Recently, lignocellulosic biomass has been viewed as a promising feedstock for the synthesis of bio-based polymeric alternatives to conventional petroleum-based macromolecules [17]. As a major component of lignocellulosic biomass, lignin is the most abundant and renewable source of potential natural aromatic chemicals [18]. Hence, lignin-derivable polymers have been regarded as greener and more sustainable materials, capable of reducing carbon dioxide and other greenhouse gas emissions [19]. In addition to sustainability considerations, the thermomechanical properties (*e.g.,* glass transition temperature, Young's modulus, tensile strength) of lignin-derivable polymers are comparable to relevant petroleum-based polymers in laboratory testing [20, 21, 22].

Bisguaiacols are one key lignin-derivable building blocks and can be readily produced from lignin deconstruction products such as hydroxybenzyl alcohols *(e.g.,* vanillyl alcohol or syringyl alcohol) and methoxyphenols (*e.g.,* phenol, guaiacol, or syringol) [23]. Furthermore, various regioisomers, such as *p,p′, m,p′,* and *o,p′*-bisguaiacols (see Figure 1), can be synthesized in an analogous manner to isomers of BPA and BPF [17]. A key difference is that bisguaiacols bear methylene bridges present in BPF but are produced without formaldehyde, whereas BPA has an isopropylene bridge [24]. Additionally, in comparison to BPF, bisguaiacol P (BGP) has one methoxy group, bisguaiacol S (BGS) has three methoxy groups, and bisguaiacol M (BGM) has four methoxy groups on the aromatic rings. It has been reported that the methoxy substituents on these bisguaiacols may mitigate the toxicity concerns that are commonly associated with BPA or BPF [25, 26].

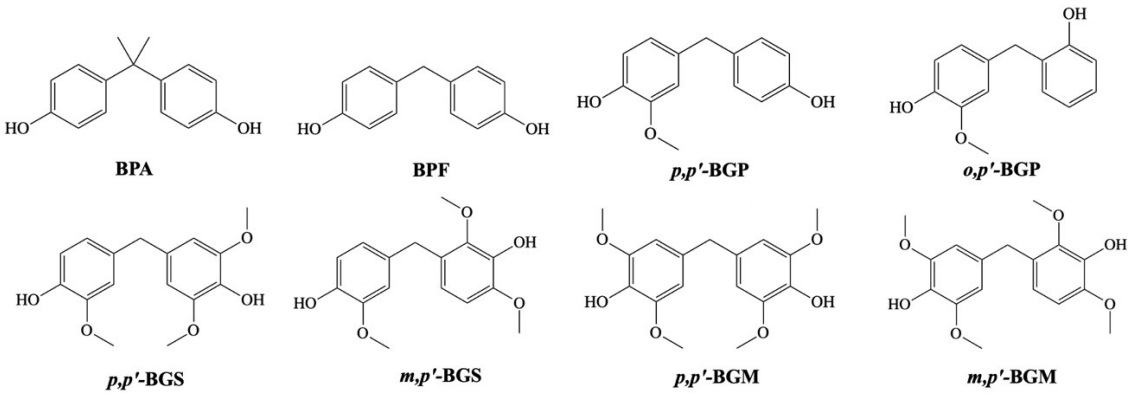

Figure 1. Chemical structures of BPA, BPF, *p,p′*-BGP, *o,p′*-BGP, *p,p′*-BGS, *m,p′*-BGS, *p,p′*-BGM, and *m,p′*-BGM.

Previous studies have reported that the six lignin-derivable bisguaiacols had undetectable EA in comparison to BPA and BPF at environmentally relevant concentrations (10^{-10} – 10^{-7} M) [27]; however, EA is not the only potential toxicity endpoint for these bisguaiacols. Considering the structural similarities between bisguaiacols and BPA/BPF, the possible genotoxicity of these new compounds should not be ignored. *In vivo* assays are one option to estimate the genotoxicity of these bisphenols as recommended in the latest guidelines of the Organization for Economic Co-operation and Development (OECD) [28]. Following the 3Rs (Replacement, Reduction, and Refinement) for using animals in toxicological studies, the chicken embryo model has been recognized as an alternative to animal studies [29]. Thus, in this study, we investigated the genotoxicity of six bisguaiacols (*p,p′*-BGP, *o,p′*-BGP, *m,p′*-BGS, *p,p′*-BGS, *m,p′*-BGM, and *p,p′*-BGM) using *in silico* simulations with the toxicity estimation software tool (T.E.S.T.), *in vitro* Ames tests, and *in vivo* comet assays, for a more comprehensive evaluation of this important toxicity endpoint. The comet assay was performed on isolated liver cells from the chicken embryos after injection of the test compound(s) on three successive days (day 7 - day 9) at 0.01 mM injection concentration, which resulted in final doses of 6.7 to 10.8 μg/kg egg weight (EW)/day. To measure various types of DNA damage, in addition to the standard strand breaks [30, 31], we used formamidopyrimidine DNA glycosylase (Fpg) in combination with the standard comet assay to detect oxidatively damaged DNA, especially oxidized purines [32]. The Fpg enzyme has been added to different cell lines to aid in genotoxicity testing [33]; however, the *in vivo* Fpg-modified assay has only been used in mice, rats, and aquatic organisms in the recent literature [23, 35, 36, 37]. The lipid peroxidation levels also were evaluated in chicken fetal liver samples concurrent

with the comet assay to determine the potential correlation between liver oxidative stress and genotoxicity.

2. Materials and methods

2.1 Chemicals and tested compounds

The six lignin-derivable BPA alternatives, *p,p′*-BGP (molecular weight [MW]: 230.2 g/mol), *o,p′*-BGP (MW: 230.2 g/mol), *m,p′*-BGS (MW: 290.2 g/mol), *p,p′*-BGS (MW: 290.2 g/mol), *m,p′*-BGM (MW: 320.2 g/mol), and *p,p′*-BGM (MW: 320.2 g/mol), tested in this work were synthesized in-house according to methods described in the literature [17]. These bisguaiacols were >99 mol% pure with respect to regioisomer content. The Trevigen Comet Assay Kit (containing LMAgarose, comet slide, lysis solution, and ethylenediamine tetraacetic acid [EDTA]) was purchased from Trevigen Inc. (Gaithersburg, MD), and Fpg protein was obtained from Sigma-Aldrich (F3174). Fpg activity calibration information was used per the supplier: one unit will cleave 50% of 0.5 pmol of double-stranded DNA oligomer substrate (8-oxoguanine−mutated) in 10 min at 25 °C. The five *Salmonella typhimurium* tester strains (TA98, TA100, TA102, TA1535, and TA1537), mitomycin C (MMC), 2-aminofluorene (2AF), 2-nitrofluorene (2NF), sodium azide, 9-aminoacridine, and 2-aminoanthracene used in the Ames test were purchased from Molecular Toxicology Inc (Boone, NC, USA). Dimethylsulfoxide (DMSO, >99.7%), ethyl methanesulfonate (EMS, 99%), potassium bromate ($KBrO_3$, 99%), phosphate-buffered saline (PBS), Hank's balanced salt solution (HBSS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 99%), bovine serum albumin (BSA), potassium chloride (KCl, >99%), and sodium hydroxide (NaOH, >95 %) were purchased from Fisher Scientific (Waltham, MA, USA). All chemicals were used as received without further purification.

2.2 T.E.S.T.

The United States Environmental Protection Agency (U.S. EPA) developed T.E.S.T. (4.2.1) for users to estimate the toxicity and physical properties of chemicals using a variety of Quantitative Structure-Activity Relationship (QSAR) methodologies, including hierarchical clustering, single model, group contribution, and nearest neighbor approaches [38]. Herein, the mutagenicity results were predicted using the consensus method, which averages the predicted toxicity values from two QSAR models (hierarchical clustering and nearest neighbor) with the

highest accuracy [39]. The structures of the test compounds (shown in Figure 1) were input into the software using the structure drawing tool. After selecting mutagenicity as the toxicity endpoint and consensus method from the QSAR list, the analysis was initiated by clicking the calculate command, and a predicted result report was generated.

2.3 Ames test

The Ames test was conducted with a preincubation method described in OECD guideline 471 (2020) with modifications on the exposure concentrations, with consideration of non-monotonic dose-response and potential human exposure levels of BPA in the environment [40]. Five *Salmonella typhimurium* tester strains TA98, TA100, TA102, TA1535, and TA1537 were used in this study. The compounds were tested in the presence of metabolic activation mixture S9 (S9 mix), as well as in the absence of S9 mix (in PBS), using liver extracts of Sprague–Dawley rats induced with Aroclor 1254. Each compound was tested at five concentrations from 10^{-8} to 10^{-4} M (0.5 pmol/plate to 5 nmol/plate) dissolved in DMSO and diluted with deionized (DI) water. The concentrations for our Ames test were derived from the possible human exposure levels of BPA in the environment. It has been reported that BPA can be detected in various environmental samples at μg/L to low mg/L [3]. Therefore, to cover this possible exposure range, we applied BPA, BPF, and the six bisguaiacols at concentrations from 10^{-8} to 10^{-4} M. For BPA (MW = 228.2 g/mol), those concentrations of BPA are 2.28 μg/L to 22.8 mg/L. A similar approach was used for unit conversion of BPF and the six bisguaiacols. One disc of lyophilized strain grew in Oxoid Nutrient Broth No.2 (20 mL) for 14 – 16 h stationary and then reached a cell density of 1×10^9 cells/mL (OD650 nm >1.0) after shaking (150 rpm) for 3 h. Volume at 0.1 mL of the bacterial cultures was mixed with 0.5 mL of S9 mix (or PBS) and 0.05 mL of test compounds at different concentrations, then incubated at 37 °C for 30 min. Next, 2 mL of molten top agar was added and poured homogeneously on the surface of minimal agar plates. The number of reverting colonies on plates was counted after 48 h of incubation at 37 °C. Mutagenicity was expressed as the number of revertants per plate with S9 mix or without S9 mix.

Both negative and positive controls were included in each assay. The negative control was 0.1% DMSO in DI water, and the positive controls were 2NF at 20 μg/plate for TA98 without S9, sodium azide at 1 μg/plate for TA100 and TA1535 without S9, MMC at 2.5 μg/plate for TA102

without S9, 9-aminoacridine at 50 μg/plate for TA1537 without S9, 2AF at 20 μg/plate for TA98, TA100, and TA102 with S9, and 2-aminoanthracene at 2 μg/plate for TA1535 and TA1537 with S9.

2.4 Comet assay

2.4.1 Standard alkaline comet assay

The standard alkaline comet assay using chicken fetal livers was performed according to Williams et al. [41], following the principles of OECD guideline 489 (2016). Fertilized Leghorn eggs were obtained from the University of Delaware (UD) research farm and were injected with BPA, BPF, and the six bisguaiacols at day 7 – day 9 before returning to incubation at 37 °C. The incubation of eggs was terminated on day 9, 3 h after the third injection. Four eggs were used for injection of each compound in every test. Livers from two randomly selected chicken embryos were pooled and processed using the following procedure. One slide with two replicates was used for each pooled liver sample. Livers were collected and rinsed with cold HBSS on ice. After the rinse, livers were transferred to 1 mL of cold HBSS containing 20 mM EDTA and 10 vol% DMSO at pH 7.5, minced into fine pieces by dissecting scissors, and allowed to settle. The cell pellets were collected after 5 min of centrifugation at 1000 rpm and dissolved in 1× PBS. Next, the cell suspensions were processed with a Trevigen Comet Assay Kit (Trevigen) according to the manufacturer's instructions [42]. Briefly, the cell suspensions were mixed with molten LMAgarose and spread onto comet slide. After the gels were solidified, the slides were placed in lysis for 14 – 16 h. Following lysis, the slides were introduced to an alkaline unwinding solution (200 mM NaOH, 1 mM EDTA, pH >13) for 1 h at 4 °C, and electrophoresed for 30 min at 25 V, 4 °C. Then, the slides were immersed twice in DI water (5 min each) followed by 70 vol% ethanol (5 min), and dried at 37 °C for 15 min. Finally, the slides were stained with SYBR Gold for 30 min in the dark and dried completely. The DNA migration on each slide was imaged using a Dragonfly microscope (Dragonfly Spinning Disk and Super Resolution Microscope) in the Bio-imaging Center at UD. The percentages of tail DNA in each image were determined using the CaspLab software (1.2.3beta2), counting >100 cells total [43].

2.4.2 Enzyme-modified comet assay

The Fpg enzyme-modified comet assay was used to assess the oxidative DNA damage, especially the 8-hydroxyguanine and ring-opened purines [44]. All the previous steps were the same as the standard alkaline assay until lysis for 14 – 16 h. Following lysis, the slides were washed three times in enzyme buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8) for 5 min each. Then, the slides were treated with Fpg enzyme or enzyme buffer (30 μL) and placed in a moisture box at 37 °C for 45 min. The Fpg stock was diluted with enzyme buffer to 0.5 μg/mL. After enzyme treatment, the slides were placed in cold alkaline unwinding solution, and all remaining steps were as described in standard comet assay. The net Fpg-sensitive sites were calculated by subtracting the tail DNA% of the enzyme buffer treatment from the tail DNA% of Fpg enzyme-treated groups.

2.5 Thiobarbituric acid reactive substance assay (TBARS assay)

The TBARS (TCA Method) Assay Kit (No. 700870) was purchased from Cayman Chemical (MI, USA) to detect the malondialdehyde (MDA) in fetal liver samples (at embryonic day 9). Briefly, 100 μL of liver tissue homogenates, 100 μL of TCA assay reagent, and 800 μL of prepared color reagent were mixed in a 1.5 mL screwcap vial. The vials were kept in boiling water for 1 h. After 1 h, the vials were placed on ice for 10 min to stop the reaction and centrifuged at 1,600 ×g at 4 °C for 10 min. Then, the MDA-TBA (thiobarbituric acid) adduct was quantified by measuring the absorbance using a microplate spectrophotometer (BioTek Synergy 2) at 530 nm, and the MDA values for samples were calculated using the standard curve of 0 - 10 μM MDA in water.

2.6 Statistical analysis

For the Ames test, the data were presented as the mean ± standard deviation (SD) of two independent tests with two replicates for each concentration. Statistical analysis was performed on a number of revertants for each strain at different compound concentrations using one-way analysis of variance (ANOVA) followed by Tukey's test in the statistical software package, JMP (JMP PRO 15) [45]. The mutagenic index (MI) was calculated as the number of revertants for the treatment sample per number of revertants for the negative control of the same strain. A mutagenic positive was identified when a two-fold increase of mutants (MI ≥ 2) was detected in at least one concentration with a dose-response relationship. Any compound with statistical significance ($p < 0.05$) as determined by its number of revertants versus the corresponding negative control, but

without an MI value higher than 2.0, was defined as having a sign of mutagenicity [46]. The graphs of these results were made by Prism GraphPad 8 software. For the comet assay and MDA results, data were calculated as mean ± SD of two independent tests with two replicates, the statistical analysis of differences among groups was assessed using one-way ANOVA followed by Dunnett's method (comparison with a control) in JMP.

3. Results and discussion

3.1 Variance toxicity results for six bisguaiacol from T.E.S.T. simulation

We first used *in silico* simulation software to evaluate the suspected mutagenicity of BPA, BPF, and the six bisguaiacols. T.E.S.T. is a toxicity simulation software developed by the U.S. EPA to generate estimates by compiling information from several QSAR models, and it has been widely employed to predict oral toxicity and mutagenicity of numerous compounds [47, 48]. For mutagenicity, the potential toxicity score can range from 0 (non-mutagen) to 1 (mutagen) [49]. Table 1 summarizes the predicted mutagenicity values of test compounds using the consensus method, in which the average predicted value from all QSAR methodologies was calculated. Chemicals with mutagenicity scores greater than or equal to 0.5 were regarded as mutagenic positive, and compounds with scores less than 0.5 were classified as mutagenic negative by the software [49]. BPA showed the lowest mutagenicity value of 0.15, followed by *o,p′*-BGP with the mutagenicity score of 0.16, whereas *m,p′*-BGS possessed the highest value of 0.72. BPF and the remaining other bisguaiacols had mutagenicity values between 0.33 and 0.49. Thus, among the eight test compounds, all lignin-derivable bisguaiacols were classified as non-mutagenic, except *m,p′*-BGS with three methoxy groups (value > 0.5) (Table 1). For each pair of stereoisomers, *m,p′* had a higher mutagenicity value than *p,p′*, and *o,p′* had a lower value than *p,p′*. However, because the accuracy of T.E.S.T. simulation results is generally ~80% [50], and because the simulation does not evaluate a dose response, further *in vitro* and *in vivo* experiments were conducted to supplement the *in silico* information.

Table 1. Number of methoxy groups and mutagenicity of BPA, BPF, and six bisguaiacol regioisomers simulated by T.E.S.T.

Compounds	Number of Methoxy groups	Mutagenicity	
		Value	Result
BPA	0	0.15	Negative
BPF	0	0.33	Negative
p,p′-BGP	1	0.33	Negative
o,p′-BGP	1	0.16	Negative
p,p′-BGS	3	0.42	Negative
m,p′-BGS	3	0.72	Positive
p,p′-BGM	4	0.35	Negative
m,p′-BGM	4	0.49	Negative

3.2 Mutagenicity assessment of six bisguaiacols by the Ames test

To confirm the *in silico* simulation outcomes, we investigated the mutagenic activity of the six bisguaiacols (at treatment concentrations: 10^{-8} to 10^{-4} M; final dosages: 0.5 pmol/plate to 5 nmol/plate) on five tester strains (TA98, TA100, TA102, TA1535, and TA1537). Even though there was a recommendation of using TA98 and TA100 alone to replace the traditional Ames test from the International Workshop on Genotoxicity Testing [51], the recently updated OECD TG 471 guideline still suggests using at least five tester strains. Therefore, by following this updated guideline, we included five tester strains in the current study to target frameshift (TA98 and TA1537), base-pair substitution (TA100 and TA1535), and transversion on nucleotides AT (TA102) [52]. The positive control of each tester strain, with or without S9 mix, produced a statistically significant increase in the number of revertants colonies vs. the negative controls (Table 2 and 3). Additionally, the revertants number of the negative controls on five tester strains were within our historical ranges, which confirmed the sensitivity and accuracy of the test system. Finally, no precipitation was detected in the Ames test at the experimental conditions employed. On the basis of the literature [53], the MI value of 2.0 was considered the critical value to determine if a test chemical was mutagenic.

Table 2. Mutagenic effects of six bisguaiacols as determined by the Ames test without S9 mix. Revertants are presented as means ± SD from two independent trials with two replicates each. MI is calculated as the number of revertants for treated samples divided by the number of revertants for the negative control.

Compounds	**Concentration (pmol/plate)**	**Final dosage (× 10^{-6} mg/plate)**	**Without S9 activation**				
			TA1535	**TA1537**	**TA98**	**TA100**	**TA102**

			revertants	MI	revertants	MI	revertants	MI	revertants	MI	revertants	MI
	5000	1150	9 ± 1	0.9	5 ± 1	0.7	10 ± 1	0.7	77 ± 5	1.1	233 ± 10	0.9
	500	115	12 ± 2	1.2	5 ± 2	0.7	11 ± 3	0.8	72 ± 4	1	240 ± 6	1
o,p′-BGP	50	11.5	7 ± 2	0.7	6 ± 3	0.9	13 ± 4	0.9	75 ± 4	1.1	250 ± 8	1
	5	1.15	10 ± 2	1	6 ± 1	0.9	16 ± 1	1.1	71 ± 5	1	257 ± 2	1
	0.5	0.115	8 ± 1	0.8	7 ± 1	1	17 ± 2	1.2	69 ± 1	1	257 ± 4	1
	5000	1150	8 ± 1	0.8	7 ± 2	1	13 ± 2	0.9	67 ± 2	1	224 ± 6	0.9
	500	115	8 ± 3	0.8	7 ± 4	1.1	16 ± 1	1.1	66 ± 2	0.9	232 ± 3	0.9
p,p′-BGP	50	11.5	9 ± 4	0.9	7 ± 4	1.1	15 ± 2	1	73 ± 4	1	232 ± 5	0.9
	5	1.15	5 ± 2	0.5	5 ± 4	0.7	17 ± 1	1.2	71 ± 2	1	234 ± 6	1
	0.5	0.115	7 ± 2	0.7	8 ± 2	1.2	18 ± 2	1.3	73 ± 3	1	242 ± 5	1
	5000	1450	6 ± 3	0.6	9 ± 4	1.4	12 ± 3	0.9	76 ± 2	1.1	244 ± 6	1
	500	145	8 ± 2	0.8	6 ± 2	0.8	19 ± 2	1.3	88 ± 6	1.3	253 ± 4	1
p,p′-BGS	50	14.5	11 ± 2	1.1	7 ± 2	1	14 ± 4	1	75 ± 4	1.1	215 ± 7	0.9
	5	1.45	9 ± 2	0.9	5 ± 3	0.8	19 ± 2	1.3	71 ± 2	1	235 ± 6	1
	0.5	0.145	8 ± 1	0.8	7 ± 2	1	18 ± 1	1.3	72 ± 3	1	263 ± 4	1.1
	5000	1450	10 ± 1	1.1	8 ± 4	1.2	23 ± 2	1.6	71 ± 3	1	246 ± 4	1
	500	145	8 ± 1	0.8	6 ± 2	0.8	24 ± 4*	1.7	73 ± 3	1	267 ± 4	1.1
m,p′-BGS	50	14.5	5 ± 3	0.5	7 ± 4	1.1	22 ± 3	1.6	72 ± 3	1	284 ± 6	1.2
	5	1.45	5 ± 2	0.5	7 ± 1	1	20 ± 3	1.4	88 ± 3	1.3	279 ± 2	1.1
	0.5	0.145	8 ± 1	0.8	6 ± 1	0.8	21 ± 1	1.5	83 ± 4	1.2	252 ± 3	1
	5000	1600	8 ± 4	0.8	9 ± 4	1.3	17 ± 1	1.2	71 ± 1	1	269 ± 13	1.1
	500	160	9 ± 1	0.9	8 ± 5	1.2	18 ± 3	1.3	74 ± 6	1.1	258 ± 11	1
p,p′-BGM	50	16	8 ± 1	0.8	10 ± 5	1.5	18 ± 4	1.3	86 ± 4	1.2	245 ± 7	1
	5	1.6	9 ± 4	0.9	7 ± 1	1	22 ± 2	1.5	77 ± 3	1.1	223 ± 4	0.9
	0.5	0.16	8 ± 1	0.8	7 ± 4	1	23 ± 3	1.6	80 ± 2	1.1	216 ± 8	0.9
	5000	1600	12 ± 1	1.3	7 ± 3	1.1	17 ± 1	1.2	75 ± 3	1.1	261 ± 8	1.1
	500	160	7 ± 3	0.7	7 ± 4	1	18 ± 5	1.3	87 ± 3	1.2	263 ± 4	1.1
m,p′-BGM	50	16	8 ± 2	0.8	9 ± 4	1.3	17 ± 4	1.2	87 ± 1	1.2	238 ± 4	1
	5	1.6	8 ± 3	0.8	6 ± 1	0.9	16 ± 2	1.1	78 ± 1	1.1	234 ± 6	1
	0.5	0.16	8 ± 1	0.8	7 ± 4	1	16 ± 1	1.1	81 ± 4	1.2	225 ± 5	0.9
Negative control[a]			10 ± 2		7 ± 2		14 ± 1		70 ± 3		246 ± 4	
Positive control			466 ± 76[b**]		158 ± 14[c**]		454 ± 8[d**]		775 ± 30[b**]		860 ± 14[e**]	

Negative control: [a] 0.1 vol% DMSO in DI water; Positive control: [b] sodium azide (1 μg/plate), [c] 9-aminoacridine (50 μg/plate), [d] 2NF (20 μg/plate), [e] MMC (2.5 μg/plate). Differences were evaluated using one-way ANOVA followed by Tukey's test, and statistical significance was indicated by $*p < 0.05$ and $**p < 0.01$ in comparison to the negative control.

The number of revertants colonies/plates of each compound, the corresponding MI value on five tester strains at five concentrations, and the negative control (at concentration = 0) without S9 mix are shown in Table 2. The revertants of the negative control group (0.1 vol% DMSO in DI water) were 10 ± 2, 7 ± 2, 14 ± 1, 70 ± 3, and 246 ± 4 for TA1535, TA1537, TA98, TA100, and TA102, respectively. The results showed that *o,p′*-BGP, *p,p′*-BGP, *p,p′*-BGS, and *m,p′*-BGM had a similar number of revertants to the negative control *S. typhimurium* strains, with the MI lower than 1.5 in the absence of S9 mix. However, *m,p′*-BGS had a higher MI of 1.7 in the TA98 strain at 0.5 nmol/plate (in the absence of S9 mix), which was statistically significant ($p < 0.05$) in comparison to the negative control. For the tests with S9 mixture (Table 3), the revertants of the negative control group (0.1 vol% DMSO in DI water) were 8 ± 1, 17 ± 5, 14 ± 4, 83 ± 4, and 264 ± 6 for TA1535, TA1537, TA98, TA100, and TA102, respectively. The *o,p′*-BGP and *p,p′*-BGP compounds had MI values of 1.4 on TA1535 (at 0.5 pmol/plate to 0.5 nmol/plate) and TA1537 (from 0.5 pmol/plate to 5 nmol/plate), respectively. The *p,p′*-BGS showed the highest MI among the six compounds at 1.8 on TA1535 (at 0.05 nmol/plate), and *m,p′*-BGS possessed an MI of 1.6 for TA98 (at 0.05 nmol/plate). The *p,p′*-BGM exhibited an MI value of 1.5 in TA1535 at 5 pmol/plate. There were no MI values greater than 1.2 for the *m,p′*-BGM. However, no significant difference existed for all compounds in the presence of S9 versus the negative control ($p < 0.05$). A non-monotonic dose response was detected for all compounds in at least one tester strain.

Table 3. Mutagenic effects of six bisguaiacols determined by the Ames test with S9 mix. Revertants are presented as means ± SD from two independent trials. MI is calculated as the number of revertants for treatment divided by the number of revertants for the negative control.

Compounds	Concentration (pmol/plate)	Final dosage ($\times 10^{-6}$ mg/plate)	With S9 activation									
			TA1535		TA1537		TA98		TA100		TA102	
			revertants	MI	revertants	MI	Revertants	MI	revertants	MI	revertants	MI
o,p′-BGP	5000	1150	10 ± 3	1.3	20 ± 1	1.2	14 ± 4	1	78 ± 4	0.9	285 ± 4	1.1
	500	115	11 ± 1	1.4	18 ± 1	1.1	17 ± 2	1.2	74 ± 1	0.9	287 ± 4	1.1
	50	11.5	11 ± 2	1.3	18 ± 3	1.1	14 ± 5	1	82 ± 3	1	282 ± 6	1.1
	5	1.15	12 ± 2	1.4	19 ± 2	1.1	16 ± 4	1.1	82 ± 2	1	280 ± 4	1.1
	0.5	0.115	9 ± 3	1.1	19 ± 3	1.2	15 ± 2	1	90 ± 3	1.1	274 ± 6	1
p,p′-BGP	5000	1150	11 ± 2	1.3	17 ± 1	1	17 ± 1	1.2	78 ± 6	0.9	283 ± 3	1.1
	500	115	8 ± 3	1	17 ± 4	1	16 ± 4	1.1	76 ± 4	0.9	276 ± 6	1
	50	11.5	10 ± 1	1.3	22 ± 5	1.3	16 ± 3	1.1	79 ± 3	1	270 ± 4	1
	5	1.15	8 ± 3	1	23 ± 5	1.4	15 ± 4	1.1	81 ± 2	1	263 ± 4	1
	0.5	0.115	11 ± 2	1.3	21 ± 1	1.3	17 ± 2	1.2	80 ± 4	1	265 ± 6	1

	5000	1450	12 ± 2	1.4	17 ± 1	1	15 ± 4	1.1	79 ± 3	1	296 ± 3	1.1
	500	145	13 ± 1	1.6	13 ± 2	0.8	20 ± 2	1.4	77 ± 4	0.9	288 ± 4	1.1
p,p′-BGS	50	14.5	15 ± 1	1.8	16 ± 2	0.9	17 ± 5	1.2	81 ± 1	1	306 ± 6	1.2
	5	1.45	12 ± 3	1.5	14 ± 2	0.8	18 ± 3	1.3	82 ± 1	1	274 ± 6	1
	0.5	0.145	12 ± 1	1.5	16 ± 2	0.9	19 ± 3	1.4	77 ± 3	0.9	286 ± 4	1.1
	5000	1450	11 ± 3	1.4	16 ± 3	1	15 ± 6	1.1	83 ± 2	1	269 ± 13	1
	500	145	12 ± 1	1.5	21 ± 4	1.2	20 ± 3	1.4	87 ± 2	1	286 ± 4	1.1
m,p′-BGS	50	14.5	9 ± 2	1.1	18 ± 2	1.1	22 ± 1	1.6	99 ± 5	1.2	293 ± 4	1.1
	5	1.45	11 ± 1	1.3	22 ± 2	1.3	17 ± 3	1.2	96 ± 8	1.2	286 ± 11	1.1
	0.5	0.145	9 ± 3	1.1	20 ± 1	1.2	19 ± 1	1.4	89 ± 3	1.1	291 ± 16	1.1
	5000	1600	10 ± 1	1.3	21 ± 2	1.2	14 ± 5	1	79 ± 1	1	292 ± 16	1.1
	500	160	12 ± 2	1.4	13 ± 1	0.8	16 ± 5	1.1	73 ± 4	0.9	311 ± 13	1.2
p,p′-BGM	50	16	11 ± 3	1.4	15 ± 2	0.9	15 ± 6	1.1	89 ± 3	1.1	288 ± 15	1.1
	5	1.6	12 ± 1	1.5	17 ± 1	1	19 ± 2	1.3	88 ± 3	1.1	270 ± 15	1
	0.5	0.16	11 ± 1	1.4	20 ± 2	1.2	15 ± 4	1.1	86 ± 4	1	260 ± 11	1
	5000	1600	9 ± 3	1.1	20 ± 2	1.2	17 ± 2	1.2	81 ± 4	1	294 ± 6	1.1
	500	160	8 ± 1	1	14 ± 3	0.8	17 ± 2	1.2	78 ± 3	0.9	284 ± 8	1.1
m,p′-BGM	50	16	10 ± 1	1.2	19 ± 1	1.2	15 ± 6	1	88 ± 5	1.1	273 ± 10	1
	5	1.6	10 ± 2	1.2	19 ± 2	1.1	13 ± 3	0.9	85 ± 4	1	265 ± 13	1
	0.5	0.16	9 ± 3	1.1	18 ± 2	1.1	15 ± 4	1	84 ± 3	1	250 ± 4	0.9
Negative control[a]			8 ± 1		17 ± 5		14 ± 4		83 ± 4		264 ± 6	
Positive control			218 ± 11[b**]		389 ± 33[b**]		687 ± 33[c**]		701 ± 30[c**]		869 ± 13[c**]	

Negative control: [a] 0.1 vol% DMSO in DI water; Positive control: [b] 2-aminoanthracene (2 μg/plate), and [c] 2AF (20 μg/plate). Differences were evaluated using one-way ANOVA followed by Tukey's test, and statistical significance was indicated by $*p < 0.05$ and $**p < 0.01$ in comparison to the negative control.

Our Ames test results suggested no mutagenicity for the six bisguaiacols because none of their MI values reached the critical value of 2.0 [53]. However, the *m,p′*-BGS showed a sign of mutagenicity with a significantly increased number of revertants compared to the negative control, which was in general agreement with the *in silico* simulation, as only the *m,p′*-BGS was classified as mutagenic positive using the T.E.S.T. software. We did not include the BPA and BPF in the Ames test as there is sufficient published data on their mutagenicity, which indicates a lack of mutagenicity for BPA and BPF in the Ames test [54, 55]. Specifically, non-mutagenic activity was reported for BPA and BPF at 4 - 500 μg/plate in *S. typhimurium* TA98 and TA100 [54]. No mutagenic activity of BPA was detected in any of the tester strains (TA97, TA98, TA100, TA102,

and TA1535) at 10 - 5000 μg/plate [55]. As the lignin-derivable bisguaiacols possess similar structures to bisphenols, and these bisguaiacols had not been tested for mutagenicity using the Ames test, our findings are the first to show that six test bisguaiacols are not mutagenic in *S. typhimurium* TA98, TA100, TA102, TA1535, and TA1537 with and without S9 metabolic activation at the tested concentrations.

3.3 Lower DNA damage level for most of the bisguaiacols than BPA using *in vivo* comet assay

In this study, two comet assays (standard alkaline version and Fpg enzyme-modified) were performed using fetal chicken livers after a three-day (day 7 - day 9) treatment of test compounds at 0.01 mM, resulting in final doses of 7.7 μg/kg EW/day, 6.7 μg/kg EW/day, 7.7 μg/kg EW/day, 9.8 μg/kg EW/day, and 10.8 μg/kg EW/day for BPA, BPF, BGPs (*p,p′* and *o,p′*), BGSs (*p,p′* and *m,p′*), and BGMs (*p,p′* and *m,p′*), respectively. The estimated human equivalent doses were calculated from the final applied doses by considering the human safety factor (÷10) and the human equivalent dose factor from the chickens (× 18.5) [46]. These exposure dosages were lower than the reference dose for human of BPA 50 μg/kg body weight (BW)/day established by the U.S. EPA and close to the potential human exposure dosage of BPA [56]. EMS and $KBrO_3$ (at 1 mM injection concentration) served as positive controls for standard alkaline comet and Fpg enzyme-modified comet assays, respectively. Two negative controls, consisting of a non-treated group and a vehicle control (VC) group (0.1 vol% DMSO), were included for both assays. In the standard alkaline comet assay, the tail DNA% for two negative controls were 6.39 ± 1.90% (non-treated group) and 8.85 ± 0.74% (VC) (shown in Figure 2). On the other hand, EMS showed the highest tail DNA% value (31.67 ± 1.76%) [$p < 0.01$], followed by the BPA (19.09 ± 4.96%) [$p < 0.05$]. The *m,p′*-BGS and *m,p′*-BGM had nearly equivalent tail DNA% values (15.44 ± 5.03% and 15.49 ± 5.49%, respectively). The tail DNA% values for BPF (13.85 ± 2.57%) and *p,p′*-BGS (14.49 ± 2.95%) were comparable. Furthermore, *p,p′*-BGP, *o,p′*-BGP, and *p,p′*-BGM showed mean values (10.45 ± 1.19% - 11.73 ± 4.02%) that were lower than the other compounds.

The tail DNA% results of Fpg-modified comet assay were calculated as net Fpg-sensitive sites by subtracting the tail DNA% of the enzyme buffer treatment from the tail DNA% of Fpg enzyme-treated groups (Figure 2). The Fpg protein concentration (at 0.5 μg/mL) applied in this study was in accordance with dose ranges from the literature and the response from the positive

control treatment [57, 58]. The tail DNA% values for BPA (17.93 ± 3.12%) and BPF (16.07 ± 1.20%) were similar to that of the positive control, $KBrO_3$ (19.66 ± 1.77%). Both BPA, BPF, and $KBrO_3$ group showed significantly higher values of Fpg-sensitive sites than the two controls (VC and non-treated group). In contrast, none of the six bisguaiacols showed increased Fpg-sensitive sites as the values of tail DNA% (3.15 ± 2.99% to 5.74 ± 4.14%) were comparable to those of the VC (2.39 ± 2.79%) and non-treated group (2.40 ± 0.46%) [Figure 2].

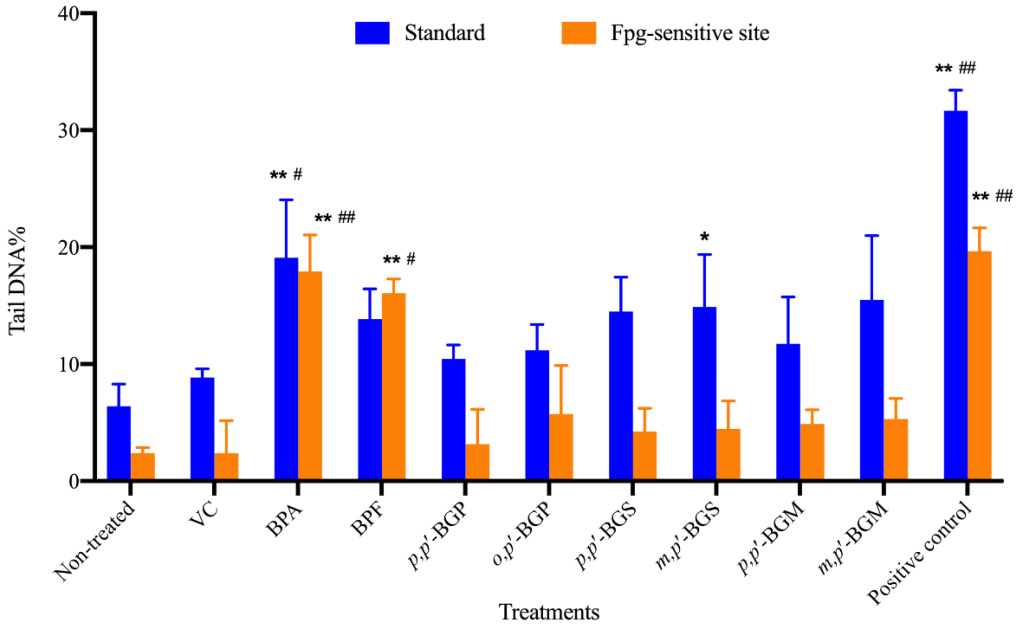

Figure 2. Comet assay results (including the standard and Fpg-modified comet assay) after a three-day (day 7 - day 9) treatment of test compounds at 0.01 mM (BPA, BPF, *p,p′*-BGP, *o,p′*-BGP, *m,p′*-BGS, *p,p′*-BGS, *m,p′*-BGM, and *p,p′*-BGM). Two control groups were included: non-treated and VC groups. The positive control for standard and Fpg-modified comet assay were EMS and $KBrO_3$, respectively. Differences were evaluated using one-way ANOVA and followed by the Dunnett's test. The statistical significance was indicated by $*p < 0.05$ and $**p < 0.01$ in comparison to the non-treated group, and $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus the VC group under each assay condition.

DNA damage is an important initial event in carcinogenesis [59]. For the first time, our study assessed DNA damage levels in the chicken fetal livers by both the standard alkaline and enzyme-modified comet assays. The standard alkaline version assesses DNA lesions that include strand

breaks and alkali-labile sites. Adding the Fpg-enzyme treatment to the standard comet assay enables this assay to detect oxidatively damaged DNA. As a DNA repair enzyme, Fpg can convert damaged bases (*e.g.*, oxidized purines) to strand breaks in DNA, and these breaks can be identified in the later procedures of the comet assay. As mentioned above, the highest and significantly increased tail DNA% (strand breaks and alkali-labile sites) was exhibited in the BPA treatment group, followed by *m,p′*-BGS and *m,p′*-BGM, with the order for the rest of the compounds as: *p,p′*-BGS > BPF > *p,p′*- BGP, *o,p′*-BGP, or *p,p′*-BGM. The standard comet assay findings are in agreement with previous genotoxicity studies on BPA and BPF in human cells [54, 58, 59]. BPA at 0.1 μmol/L to 10 μmol/L induced significant DNA damage after a 24 h exposure with no dose-response relationship in the human hepatoma cells (HepG2), whereas BPF did not induce such an increase in DNA damage [54]. DNA damage also was detected by the *in vivo* alkaline comet assay in liver tissues of female rat offspring at three different pubertal periods after BPA treatment (at 0.5 and 50 mg of BPA/kg), and in thyroid tissue from rats after 35 days of BPA treatment at 200 mg/kg [58, 59]. Furthermore, the oxidative damage to DNA pyrimidines and purines was identified by the alkaline comet test with DNA repair enzymes (endonuclease III and human 8-oxoguanine DNA glycosylase) after exposure to BPA, BPAF, BPS, and BPF in human peripheral blood mononuclear cells from 0.001 to 1 mg/mL at two exposure times (4 and 48 h) [62].

Most notably, our study revealed that the six bisguaiacols showed significantly lower levels of oxidative DNA damage in the Fpg-modified comet assay in comparison to BPA and BPF. After Fpg-enzyme treatment, BPA and BPF produced increased oxidative DNA damage, but most of the bisguaiacols showed a comparable number of Fpg-sensitive sites with the VC group at < ~5% tail DNA% (Figure 2). The *o,p′*-BGP treated group had the highest level of Fpg-sensitive sites among the six compounds at 5.74 ± 4.14%, which was still much lower than the BPA (17.93 ± 3.12%) and BPF (16.07 ± 1.20%). The lower oxidative DNA damage of lignin-derivable bisguaiacols versus BPA or BPF is mainly attributed to the methoxy groups on the aromatic rings. It has been demonstrated that natural phenolic compounds and bulk lignin with *ortho*-methoxy groups have electron-donating ability with higher antioxidant activity and DNA damage-protective effects [61, 62]. Additionally, our study suggested that increasing the number of methoxy groups on the aromatic moieties led to only a slight effect on oxidative DNA damage levels. Thus, there was no major difference between the bisguaiacols with different numbers of methoxy groups on the

oxidative DNA damage noted in the comet test. DNA damage detected by the comet assay and gene mutation revealed from the Ames test are both essential genotoxic endpoints but differ from each other. DNA damage is a structural change, usually as single or double-strand breaks, whereas mutation is a change in the nucleotide sequence of DNA. Therefore, a single test usually is not sufficient to thoroughly investigate the genotoxicity of the test compounds [65]. In this study, we applied an *in vitro* Ames test and an *in vivo* comet assay to probe gene mutation and DNA damage, respectively. Our results showed that most bisguaiacols did not exhibit any genotoxicity under the applied current experimental conditions.

3.4 TBARS assay

During chicken embryo growth, chorioallantoic respiration turns into pulmonary respiration and accelerates oxidative metabolism [66]. Increased oxidative stress can lead to numerous disorders and even mortality of chicken embryos [67]. The MDA level, which served as an essential biomarker for oxidative stress, was measured in fetal livers after a three-day treatment (at 0.01 mM) of BPA, BPF, *p,p′*-BGP, *o,p′*-BGP, *m,p′*-BGS, *p,p′*-BGS, *m,p′*-BGM, *p,p′*-BGM, and $KBrO_3$. As shown in Figure 3, the VC group and the non-treated group had MDA values of 70.62 ± 8.04 and 70.90 ± 3.04 nmol/g, respectively. The treatment groups showed relatively higher MDA values ranging from 73.23 ± 7.42 to 94.34 ± 22.15 nmol/g, but without a significant difference ($p > 0.05$).

The pro-oxidant activity of BPA and its commercial analogues has been regarded as one critical mechanism that results in adverse effects in humans and other animals. Exposure to BPA at 200 ppm on day 4 induced considerably higher MDA levels in chicken embryos, though the lower BPA concentration (50 ppm) group had no such impact [68]. Substantial levels of MDA and hydrogen peroxide also have been demonstrated in rats after exposure to BPA at 25 mg/kg/day [69, 70]. In addition to the MDA biomarker, increased oxidative stress and decreased antioxidant enzyme activities were detected in the hepatic tissue of female rat offspring exposed to BPA at 0.5 and 50 mg/kg/day [60]. In comparison to other literature [68, 69, 70], our study did not exhibit a significantly increased MDA level in the BPA treatment, which might be attributed to the varying dosage levels and durations in different animal models. Most of the literature reports [58, 67, 68] on BPA showed significantly different MDA levels in rats or mice models with much higher

dosages (around 25 - 50 mg/kg) than the dose applied in our work (7.7 μg/kg). One of the critical mechanisms of genotoxicity induced by BPA and BPF is the increased cellular oxidative stress and generation of reactive species, especially quinones, during their biotransformation, which react with DNA and cause DNA damage [16, 54]. Although the MDA levels were not significantly different between bisguaiacols and bisphenols (BPA and BPF), other oxidative pathways could be involved in pro-oxidant activities. In the future, additional oxidative stress biomarkers (*e.g.,* ROS, mitochondrial membrane potential, antioxidant enzymes levels) can be included in the chicken embryo model for a more comprehensive evaluation of oxidative stresses and their associations with genotoxicity.

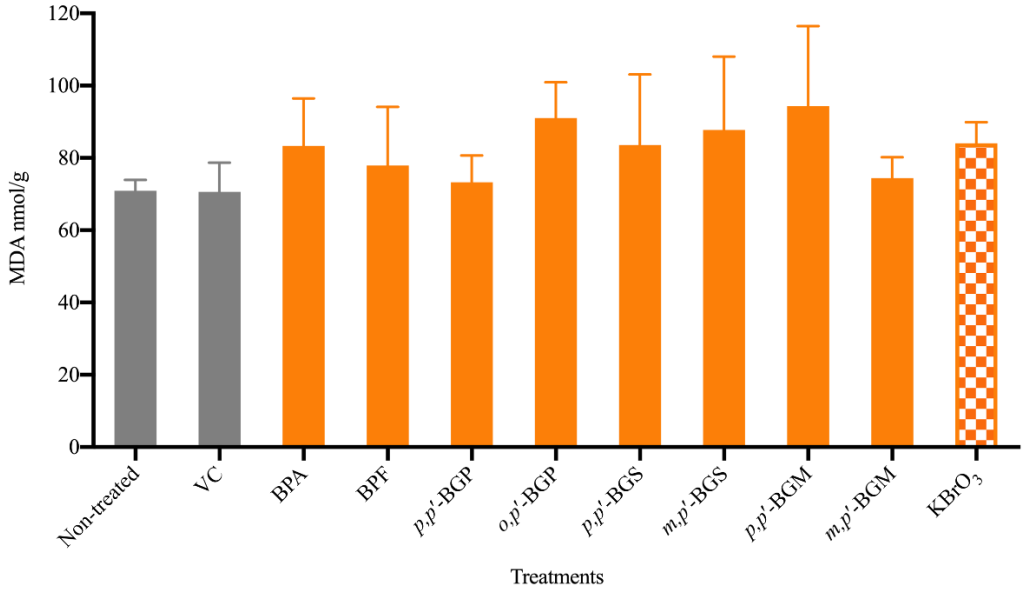

Figure 3. MDA values of fetal liver samples on day 9 after treatment with BPA, BPF, *p,p′*-BGP, *o,p′*-BGP, *m,p′*-BGS, *p,p′*-BGS, *m,p′*-BGM, *p,p′*-BGM, and $KBrO_3$. All values are expressed as mean ± SD from two independent trials.

4. Conclusion

In this work, the genotoxicity of six lignin-derivable bisguaiacols, BPA, and BPF was assessed by an *in silico* T.E.S.T. tool, *in vitro* Ames test, and *in vivo* comet assay to probe mutagenicity and DNA damage. First, *in silico* results revealed that all bisguaiacols were non-mutagenic, except for *m,p′*-BGS. Second, *in vitro* Ames test suggested no bisguaiacols had

mutagenicity in five tester strains. Building upon those conclusions, two forms of the comet assay (standard alkaline and Fpg enzyme-modified) were applied to chicken embryo models. In the standard alkaline assay, except for *m,p′*-BGS, the other five bisguaiacols and BPF did not induce increased DNA damage in comparison to BPA and EMS. In the Fpg enzyme-modified comet assay, all six bisguaiacols did not induce oxidative DNA damage. Conversely, BPA and BPF showed a significantly higher number of Fpg-sensitive sites versus the non-treated group (or VC group). Furthermore, negative results were noted from TBARS assay in fetal livers after a three-day treatment. Overall, these findings suggest that having at least one methoxy ortho to a phenolic hydroxyl group contributed to the lower oxidative DNA damage in comparison with BPA and BPF (*i.e.,* no methoxy groups). Additionally, the increased number of methoxy groups on bisguaiacols had a minor impact on oxidative genotoxicity. In summary, the six lignin-derivable bisguaiacols showed fewer concerns with respect to genotoxicity.

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Disclaimer

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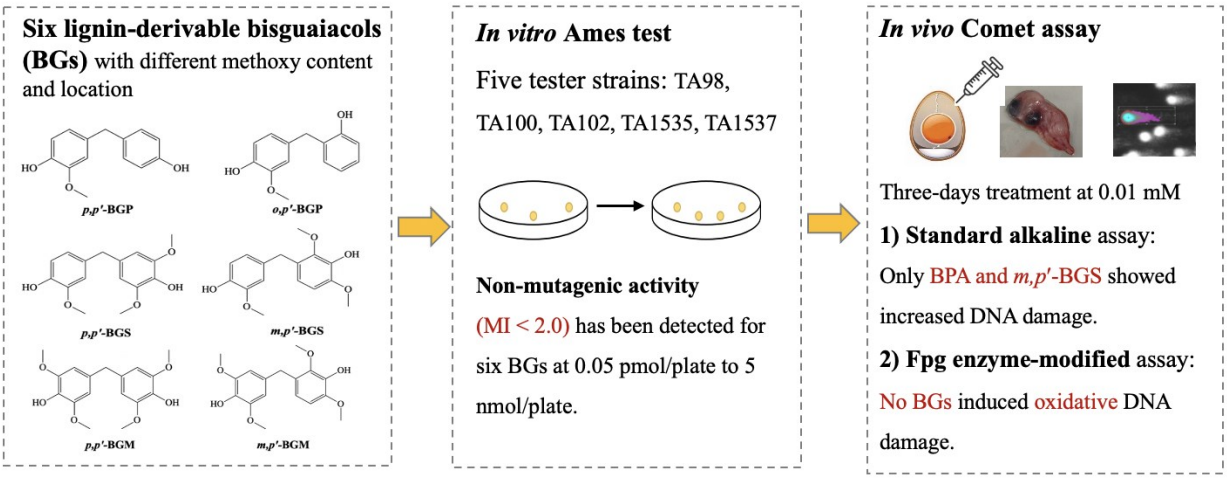
Graphical abstract



References

[1] J.S. Mahajan, R.M. O'Dea, J.B. Norris, L.T.J. Korley, T.H. Epps, III, Aromatics from Lignocellulosic Biomass: A Platform for High-Performance Thermosets, ACS Sustain. Chem. Eng. 8 (2020) 15072–15096.

[2] L. Trullemans, S.-F. Koelewijn, I. Scodeller, T. Hendrickx, P. Van Puyvelde, B.F. Sels, A guide towards safe, functional and renewable BPA alternatives by rational molecular design: structure–property and structure–toxicity relationships, Polym. Chem. 12 (2021) 5870–5901.

[3] R.M. O'Dea, J.A. Willie, T.H. Epps, III, 100th anniversary of macromolecular science viewpoint: polymers from lignocellulosic biomass. Current challenges and future opportunities, ACS Macro Lett. 9 (2020) 476–493.

[4] H.-J. Lehmler, B. Liu, M. Gadogbe, W. Bao, Exposure to bisphenol A, bisphenol F, and bisphenol S in US adults and children: The national health and nutrition examination survey 2013–2014, ACS Omega. 3 (2018) 6523–6532.

[5] F. Liguori, C. Moreno-Marrodan, P. Barbaro, Biomass-derived chemical substitutes for bisphenol A: recent advancements in catalytic synthesis, Chem. Soc. Rev. 49 (2020) 6329–6363.

[6] C. Rowell, N. Kuiper, H. Preud'Homme, Is container type the biggest predictor of trace element and BPA leaching from drinking water bottles?, Food Chem. 202 (2016) 88–93.

[7] M.R. Bernier, L.N. Vandenberg, Handling of thermal paper: Implications for dermal exposure to bisphenol A and its alternatives, PLoS One. 12 (2017) e0178449.

[8] K. Mikołajewska, J. Stragierowicz, J. Gromadzińska, Bisphenol A–Application, sources of exposure and potential risks in infants, children and pregnant women, Int. J. Occup. Med. Environ. Health. 28 (2015) 209–241.

[9] N.S. Graziani, H. Carreras, E. Wannaz, Atmospheric levels of BPA associated with particulate matter in an urban environment, Heliyon. 5 (2019) e01419.

[10] Y. Ma, H. Liu, J. Wu, L. Yuan, Y. Wang, X. Du, R. Wang, P.W. Marwa, P. Petlulu, X. Chen, The adverse health effects of bisphenol A and related toxicity mechanisms, Environ.

Res. 176 (2019) 108575.
[11] J. Lee, S. Park, J. Byun, M. Lee, Y.-S. Do, Y. Kim, M. Kwon, Distribution and Potential Transdermal Human Intake of Bisphenol A and Bisphenol S from Thermal Receipt Papers in Korea Market, Expo. Heal. 13 (2021) 477–485.
[12] N. Cabaton, C. Dumont, I. Severin, E. Perdu, D. Zalko, M. Cherkaoui-Malki, M.C. Chagnon, Genotoxic and endocrine activities of bis(hydroxyphenyl)methane (bisphenol F) and its derivatives in the HepG2 cell line, Toxicology. 255 (2009) 15–24.
[13] B. Lei, S. Sun, X. Zhang, C. Feng, J. Xu, Y. Wen, Y. Huang, M. Wu, Y. Yu, Bisphenol AF exerts estrogenic activity in MCF-7 cells through activation of Erk and PI3K/Akt signals via GPER signaling pathway, Chemosphere. 220 (2019) 362–370.
[14] V. Le Fol, S. Aït-Aïssa, M. Sonavane, J.-M. Porcher, P. Balaguer, J.-P. Cravedi, D. Zalko, F. Brion, In vitro and in vivo estrogenic activity of BPA, BPF and BPS in zebrafish-specific assays, Ecotoxicol. Environ. Saf. 142 (2017) 150–156.
[15] S. Ikhlas, A. Usman, M. Ahmad, In vitro study to evaluate the cytotoxicity of BPA analogues based on their oxidative and genotoxic potential using human peripheral blood cells, Toxicol. Vitr. 60 (2019) 229–236.
[16] K. Hercog, S. Maisanaba, M. Filipič, M. Sollner-Dolenc, L. Kač, B. Žegura, Genotoxic activity of bisphenol A and its analogues bisphenol S, bisphenol F and bisphenol AF and their mixtures in human hepatocellular carcinoma (HepG2) cells, Sci. Total Environ. 687 (2019) 267–276.
[17] K.H. Nicastro, C.J. Kloxin, T.H. Epps, III, Potential lignin-derived alternatives to bisphenol a in diamine-hardened epoxy resins, ACS Sustain. Chem. Eng. 6 (2018) 14812–14819.
[18] L.C. Over, E. Grau, S. Grelier, M.A.R. Meier, H. Cramail, Synthesis and characterization of epoxy thermosetting polymers from glycidylated organosolv lignin and Bisphenol a, Macromol. Chem. Phys. 218 (2017) 1600411.
[19] G.F. Bass, T.H. Epps, III, Recent developments towards performance-enhancing lignin-based polymers, Polym. Chem. 12 (2021) 4130–4158.
[20] A.L. Holmberg, K.H. Reno, R.P. Wool, T.H. Epps, III, Biobased building blocks for the rational design of renewable block polymers, Soft Matter. 10 (2014) 7405–7424.
[21] K.M. Hambleton, J.F. Stanzione III, Synthesis and Characterization of a Low-Molecular-Weight Novolac Epoxy Derived from Lignin-Inspired Phenolics, ACS Omega. 6 (2021) 23855–23861.
[22] A. Pellis, J.W. Comerford, S. Weinberger, G.M. Guebitz, J.H. Clark, T.J. Farmer, Enzymatic synthesis of lignin derivable pyridine based polyesters for the substitution of petroleum derived plastics, Nat. Commun. 10 (2019) 1–9.
[23] S.-F. Koelewijn, D. Ruijten, L. Trullemans, T. Renders, P. Van Puyvelde, H. Witters, B.F. Sels, Regioselective synthesis, isomerisation, in vitro oestrogenic activity, and copolymerisation of bisguaiacol F (BGF) isomers, Green Chem. 21 (2019) 6622–6633.
[24] E.D. Hernandez, A.W. Bassett, J.M. Sadler, J.J. La Scala, J.F. Stanzione, Synthesis and Characterization of Bio-based Epoxy Resins Derived from Vanillyl Alcohol, ACS Sustain. Chem. Eng. 4 (2016) 4328–4339.
[25] C. Wu, Y. Peng, Evaluation of Toxicity and Endocrine Disruption Potential of the Natural and Bio-Based Antimicrobials, in: Nat. Bio-Based Antimicrob. Food Appl., ACS Publications, 2018: pp. 223–241.
[26] A. Amitrano, J.S. Mahajan, L.T.J. Korley, T.H. Epps, III, Estrogenic activity of lignin-derivable alternatives to bisphenol A assessed via molecular docking simulations, RSC Adv.

11 (2021) 22149–22158.
[27] Y. Peng, K.H. Nicastro, T.H. Epps, III, C. Wu, Methoxy groups reduced the estrogenic activity of lignin-derivable replacements relative to bisphenol A and bisphenol F as studied through two in vitro assays, Food Chem. 338 (2020) 127656.
[28] OECD, Test No. 489: In Vivo Mammalian Alkaline Comet Assay, OECD Publ. (2016) Section 4.
[29] B.B. Fonseca, M.V. da Silva, L.N. de Morais Ribeiro, The chicken embryo as an in vivo experimental model for drug testing: Advantages and limitations, Lab Anim. (NY). (2021) 1–2.
[30] M. LLana-Ruiz-Cabello, S. Maisanaba, M. Puerto, A.I. Prieto, S. Pichardo, Á. Jos, A.M. Cameán, Evaluation of the mutagenicity and genotoxic potential of carvacrol and thymol using the Ames Salmonella test and alkaline, Endo III-and FPG-modified comet assays with the human cell line Caco-2, Food Chem. Toxicol. 72 (2014) 122–128.
[31] D. Muruzabal, A. Collins, A. Azqueta, The enzyme-modified comet assay: Past, present and future, Food Chem. Toxicol. (2020) 111865.
[32] A. Azqueta, L. Arbillaga, A. López de Cerain, A. Collins, Enhancing the sensitivity of the comet assay as a genotoxicity test, by combining it with bacterial repair enzyme FPG, Mutagenesis. 28 (2013) 271–277.
[33] N. El Yamani, A.R. Collins, E. Rundén-Pran, L.M. Fjellsbø, S. Shaposhnikov, S. Zienolddiny, M. Dusinska, In vitro genotoxicity testing of four reference metal nanomaterials, titanium dioxide, zinc oxide, cerium oxide and silver: towards reliable hazard assessment, Mutagenesis. 32 (2017) 117–126.
[34] N. Asare, N. Duale, H.H. Slagsvold, B. Lindeman, A.K. Olsen, J. Gromadzka-Ostrowska, S. Meczynska-Wielgosz, M. Kruszewski, G. Brunborg, C. Instanes, Genotoxicity and gene expression modulation of silver and titanium dioxide nanoparticles in mice, Nanotoxicology. 10 (2016) 312–321.
[35] P. Jalili, S. Huet, R. Lanceleur, G. Jarry, L. Le Hegarat, F. Nesslany, K. Hogeveen, V. Fessard, Genotoxicity of aluminum and aluminum oxide nanomaterials in rats following oral exposure, Nanomaterials. 10 (2020) 305.
[36] V. Pellegri, G. Gorbi, A. Buschini, DNA damage detection by Comet Assay on Daphnia magna: Application in freshwater biomonitoring, Sci. Total Environ. 705 (2020) 135780.
[37] S. Kolarević, K. Sunjog, M. Kračun-Kolarević, J. Kostić-Vuković, J. Jovanović, P. Simonović, V. Simić, M. Piria, Z. Gačić, M. Lenhardt, The genetic variability (RAPD) and genotoxicity in vivo (alkaline and Fpg-modified comet assay) in chub (Squalius cephalus): The Sava River Case Study, Int. J. Environ. Res. 12 (2018) 703–712.
[38] X. Zhang, D.F. Levia, E.O. Ebikade, J. Chang, D.G. Vlachos, C. Wu, The impact of differential lignin S/G ratios on mutagenicity and chicken embryonic toxicity, J. Appl. Toxicol. 42 (2021) 423–435.
[39] X. Ao, W. Sun, S. Li, C. Yang, C. Li, Z. Lu, Degradation of tetracycline by medium pressure UV-activated peroxymonosulfate process: influencing factors, degradation pathways, and toxicity evaluation, Chem. Eng. J. 361 (2019) 1053–1062.
[40] OECD, Guideline for testing of chemicals Test No.471: Bacterial Reverse Mutation Test, (2020) 24.
[41] G.M. Williams, J.-D. Duan, K.D. Brunnemann, M.J. Iatropoulos, E. Vock, U. Deschl, Chicken fetal liver DNA damage and adduct formation by activation-dependent DNA-reactive carcinogens and related compounds of several structural classes, Toxicol. Sci. 141

(2014) 18–28.
[42] U. Swain, K.S. Rao, Study of DNA damage via the comet assay and base excision repair activities in rat brain neurons and astrocytes during aging, Mech. Ageing Dev. 132 (2011) 374–381.
[43] A. Osipov, E. Arkhangelskaya, A. Vinokurov, N. Smetanina, A. Zhavoronkov, D. Klokov, DNA comet Giemsa staining for conventional bright-field microscopy, Int. J. Mol. Sci. 15 (2014) 6086–6095.
[44] M. Dušinská, A. Collins, Detection of oxidised purines and UV-induced photoproducts in DNA of single cells, by inclusion of lesion-specific enzymes in the comet assay, Altern. to Lab. Anim. 24 (1996) 405–411.
[45] J. Sall, M.L. Stephens, A. Lehman, S. Loring, JMP start statistics: a guide to statistics and data analysis using JMP, Sas Institute, 2017.
[46] X. Zhang, Y. Peng, C. Wu, Chicken embryonic toxicity and potential in vitro estrogenic and mutagenic activity of carvacrol and thymol in low dose/concentration, Food Chem. Toxicol. (2021) 112038.
[47] M. Dou, J. Wang, B. Gao, C. Xu, F. Yang, Photocatalytic difference of amoxicillin and cefotaxime under visible light by mesoporous g-C3N4: mechanism, degradation pathway and DFT calculation, Chem. Eng. J. 383 (2020) 123134.
[48] C. Dang, F. Sun, H. Jiang, T. Huang, W. Liu, X. Chen, H. Ji, Pre-accumulation and in-situ destruction of diclofenac by a photo-regenerable activated carbon fiber supported titanate nanotubes composite material: Intermediates, DFT calculation, and ecotoxicity, J. Hazard. Mater. 400 (2020) 123225.
[49] US EPA, User's Guide for TEST (version 5.1)(Toxicity Estimation Software Tool): A Program to Estimate Toxicity from Molecular Structure, Chem. Charact. Expo. Div. Cincinnati O, Ed. (2020).
[50] N.G. Bakhtyari, G. Raitano, E. Benfenati, T. Martin, D. Young, Comparison of in silico models for prediction of mutagenicity, J. Environ. Sci. Heal. - Part C Environ. Carcinog. Ecotoxicol. Rev. 31 (2013) 45–66.
[51] R. V Williams, D.M. DeMarini, L.F. Stankowski Jr, P.A. Escobar, E. Zeiger, J. Howe, R. Elespuru, K.P. Cross, Are all bacterial strains required by OECD mutagenicity test guideline TG471 needed?, Mutat. Res. Toxicol. Environ. Mutagen. 848 (2019) 503081.
[52] U. Vijay, S. Gupta, P. Mathur, P. Suravajhala, P. Bhatnagar, Microbial Mutagenicity Assay: Ames Test, Biol. Protoc. 8 (2018) 32763.
[53] F.A. Resende, W. Vilegas, L.C. Dos Santos, E.A. Varanda, Mutagenicity of flavonoids assayed by bacterial reverse mutation (Ames) test, Molecules. 17 (2012) 5255–5268.
[54] A. Fic, B. Žegura, M.S. Dolenc, M. Filipič, L.P. Mašič, Mutagenicity and DNA damage of bisphenol A and its structural analogues in HepG2 cells, Arch. Ind. Hyg. Toxicol. 64 (2013) 189–200.
[55] L. Xin, Y. Lin, A. Wang, W. Zhu, Y. Liang, X. Su, C. Hong, J. Wan, Y. Wang, H. Tian, Cytogenetic evaluation for the genotoxicity of bisphenol-A in Chinese hamster ovary cells, Environ. Toxicol. Pharmacol. 40 (2015) 524–529.
[56] Z. Wang, H. Liu, S. Liu, Low-dose bisphenol A exposure: A seemingly instigating carcinogenic effect on breast cancer, Adv. Sci. 4 (2017) 1600248.
[57] Y. Guichard, M. Maire, S. Sébillaud, C. Fontana, C. Langlais, J. Micillino, C. Darne, J. Roszak, M. Stępnik, V. Fessard, Genotoxicity of synthetic amorphous silica nanoparticles in rats following short-term exposure, part 2: intratracheal instillation and intravenous

injection, Environ. Mol. Mutagen. 56 (2015) 228–244.
[58] C.S. Chen, Y.C. Hseu, S.H. Liang, J.-Y. Kuo, S.C. Chen, Assessment of genotoxicity of methyl-tert-butyl ether, benzene, toluene, ethylbenzene, and xylene to human lymphocytes using comet assay, J. Hazard. Mater. 153 (2008) 351–356.
[59] J.L. Barnes, M. Zubair, K. John, M.C. Poirier, F.L. Martin, Carcinogens and DNA damage, Biochem. Soc. Trans. 46 (2018) 1213–1224.
[60] J.I. Eid, S.M. Eissa, A.A. El-Ghor, Bisphenol A induces oxidative stress and DNA damage in hepatic tissue of female rat offspring, J. Basic Appl. Zool. 71 (2015) 10–19.
[61] E.T. Mohammed, K.S. Hashem, A.E. Ahmed, M.T. Aly, L. Aleya, M.M. Abdel-Daim, Ginger extract ameliorates bisphenol A (BPA)-induced disruption in thyroid hormones synthesis and metabolism: Involvement of Nrf-2/HO-1 pathway, Sci. Total Environ. 703 (2020) 134664.
[62] K. Mokra, K. Woźniak, B. Bukowska, P. Sicińska, J. Michałowicz, Low-concentration exposure to BPA, BPF and BPAF induces oxidative DNA bases lesions in human peripheral blood mononuclear cells, Chemosphere. 201 (2018) 119–126.
[63] L.-L. Zhang, L.-F. Zhang, J.-G. Xu, Q.-P. Hu, Comparison study on antioxidant, DNA damage protective and antibacterial activities of eugenol and isoeugenol against several foodborne pathogens, Food Nutr. Res. 61 (2017) 1353356.
[64] C. Hu, Y. V Yuan, D.D. Kitts, Antioxidant activities of the flaxseed lignan secoisolariciresinol diglucoside, its aglycone secoisolariciresinol and the mammalian lignans enterodiol and enterolactone in vitro, Food Chem. Toxicol. 45 (2007) 2219–2227.
[65] D. Sun, T. Zhao, T. Wang, M. Wu, Z. Zhang, Genotoxicity assessment of triclocarban by comet and micronucleus assays and Ames test, Environ. Sci. Pollut. Res. 27 (2020) 7430–7438.
[66] X. Xiao, D. Yuan, Y.-X. Wang, X.-A. Zhan, The protective effects of different sources of maternal selenium on oxidative stressed chick embryo liver, Biol. Trace Elem. Res. 172 (2016) 201–208.
[67] K. Li, L. Jiang, J. Wang, L. Xia, R. Zhao, C. Cai, P. Wang, X. Zhan, Y. Wang, Maternal dietary supplementation with different sources of selenium on antioxidant status and mortality of chicken embryo in a model of diquat-induced acute oxidative stress, Anim. Feed Sci. Technol. 261 (2020) 114369.
[68] S. Gharibi, A. Dilmaghanian, P. Sadighara, R.M. Nezhad Fard, A. Erfanmanesh, T. Mohajerfar, T. Farkhondeh, The effect of bisphenol a on oxidative stress indices and pathological changes in the brain of chicken embryos, World Appl. Sci. J. 26 (2013) 345–351.
[69] H. Kabuto, M. Amakawa, T. Shishibori, Exposure to bisphenol A during embryonic/fetal life and infancy increases oxidative injury and causes underdevelopment of the brain and testis in mice, Life Sci. 74 (2004) 2931–2940.
[70] B. Avci, A. Bahadir, O.K. Tuncel, B. Bilgici, Influence of α-tocopherol and α-lipoic acid on bisphenol-A-induced oxidative damage in liver and ovarian tissue of rats, Toxicol. Ind. Health. 32 (2016) 1381–1390.