Lignin-Derivable Alternatives to Bisphenol A with Potentially Undetectable Estrogenic Activity and Minimal Developmental Toxicity

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Abstract

Lignin-derivable bisguaiacols/bissyringols are viable alternatives to commercial bisphenols; however, many bisguaiacols/bissyringols (*e.g.*, bisguaiacol F [BGF]) have unsubstituted bridging carbons between the aromatic rings, making them more structurally similar to bisphenol F (BPF) than bisphenol A (BPA) – both of which are suspected endocrine disruptors. Herein, we investigated the estrogenic activity (EA) and developmental toxicity of dimethyl-substituted bridging carbon-based lignin-derivable bisphenols (bisguaiacol A [BGA] and bissyringol A [BSA]). Notably, BSA showed undetectable EA at seven test concentrations (from 10^{-12} M to 10^{-6} M) in the MCF-7 cell proliferation assay, whereas BPA had detectable EA at five concentrations (from 10^{-10} M to 10^{-6} M). *In silico* results indicated that BSA had the lowest binding affinity with estrogen receptors. Moreover, *in vivo* chicken embryonic assay results revealed that lignin-derivable monomers had minimal developmental toxicity vs. BPA at environmentally relevant test concentrations (8.7 to 116 μg/kg). Additionally, all lignin-derivable compounds showed significantly lower expression fold changes (from ~1.81 to ~4.41) in chicken fetal liver for an estrogen-response gene (apolipoprotein II) in comparison to BPA (fold change of ~11.51), which was indicative of significantly reduced estrogenic response. Altogether, the methoxy substituents on lignin-derivable bisphenols appeared to be a positive factor in reducing EA of BPA alternatives.

Keywords: Lignin-derivable; Bio-based; Bisphenol A replacement; Estrogenic activity; Developmental toxicity; Chicken embryo

1. Introduction

Bisphenol A (BPA) is an essential building block for many polymeric systems, including polycarbonates, polysulfones, and epoxy resins, and these materials are widely used in applications such as food contact materials (FCMs) (Trullemans et al., 2021; Mahajan et al., 2020). Due to the adverse health effects associated with BPA, such as endocrine disruption, genotoxicity, and reproductive toxicity, coupled with heightened human exposure, many countries have implemented restrictions on the use of BPA in FCMs, especially in products designed for infants and children (Vom Saal et al., 2012; Ďurovcová et al., 2022; Yin et al., 2017; Chen et al., 2015). For example, the use of BPA in baby bottles was banned in Canada in 2008 (Erler and Novak, 2010), and the European Commission banned BPA usage in coatings of infant-related packaging in

2011 (Usman and Ahmad, 2019). The United States (U.S.) Food and Drug Administration (FDA) took similar steps to ban the use of BPA in infant-associated packaging in 2013 (Usman and Ahmad, 2019). In addition to the toxicity concerns, BPA is derived from petrochemical feedstocks, which are limited in quantity, unevenly distributed globally, subject to various environmental concerns (Bass and Epps, III, 2021). Consequently, there is a growing demand for less toxic and more sustainable alternatives to BPA.

To meet the increased demand for "BPA-free" products, a variety of BPA alternatives have emerged, including bisphenol F (BPF), bisphenol S (BPS), bisphenol AF (BPAF), *etc*. These commercial alternatives are usually petroleum-derived and possess structural similarities to BPA, and therefore raise similar long-term sustainability and toxicological concerns (Moreman et al., 2017; Qiu et al., 2019; Lei et al., 2017). These alternative bisphenols also possess an endocrine disruption effect (EDE), and they have been classified as endocrine-disrupting chemicals (EDCs) (Lei et al., 2017). Moreover, the developmental toxicity of these commercial bisphenols has been reported as a rising health issue (Harnett et al., 2021; Mu et al., 2018; Yin et al., 2019). Considering the health concerns associated with commercial bisphenols, it is imperative to develop alternative BPA replacements that exhibit reduced toxicity.

Lignin is the most abundant potential source of renewable aromatic chemicals (Mahajan et al., 2020; Bass and Epps, III, 2021; O'Dea et al., 2020; Schutyser et al., 2018; Cywar et al., 2022; Nicastro et al., 2018). Bulk lignin can be deconstructed into several substituted phenols and can be further converted to bisguaiacols/bissyringols (O'Dea et al., 2020; Schutyser et al., 2018; Mahajan et al., 2020). These lignin-derivable bisphenols are suggested as safer alternatives to commercial counterparts; the methoxy groups on these bisguaiacols/bissyringols are believed to hinder the binding of phenolic hydroxyls with estrogen receptors, contributing to their safety profile (Amitrano et al., 2021). Peng et al. reported that three bisguaiacol F (BGF) mixtures with different regioisomer contents showed lower estrogenic activity (EA) than BPA in two *in vitro* assays on the basis of breast cancer cell lines (MCF-7) (Peng et al., 2018). Apart from BGF, other bisguaiacols (bisguaiacol P [BGP], bisguaiacol S [BGS], and bisguaiacol M [BGM]) with varying degrees of methoxy substitution have demonstrated lower EA, genotoxicity, and oxidative DNA damage when compared to BPA (Peng et al., 2020; Zhang et al., 2022). BGF/BGM/BGP/BGS

possess an unsubstituted bridging carbon between the aromatic rings, and the absence of substituents on the bridging carbon in these bisguaiacols allows free rotation of the resultant polymer backbone that led to the lower glass transition temperatures (T_gs) vs. those of BPA analogues. But, by incorporating dimethyl substituents on the bridging carbon, the T_gs of lignin-derivable polymeric systems become comparable to those of the BPA counterparts (Mhatre et al., 2023). Additionally, polymers containing these lignin-derivable building blocks can enhance the mechanical properties. For example, lignin-derivable non-isocyanate polyurethanes (NIPUs) have been reported with improved toughness relative to petroleum-based NIPUs (Mhatre et al., 2023). Nevertheless, it is equally important to consider the toxicity potential of the dimethyl-substituted, bridging carbon-based, lignin-derivable bisphenols (bisguaiacol A [BGA] and bissyringol A [BSA]) (Epps, III et al., 2022).

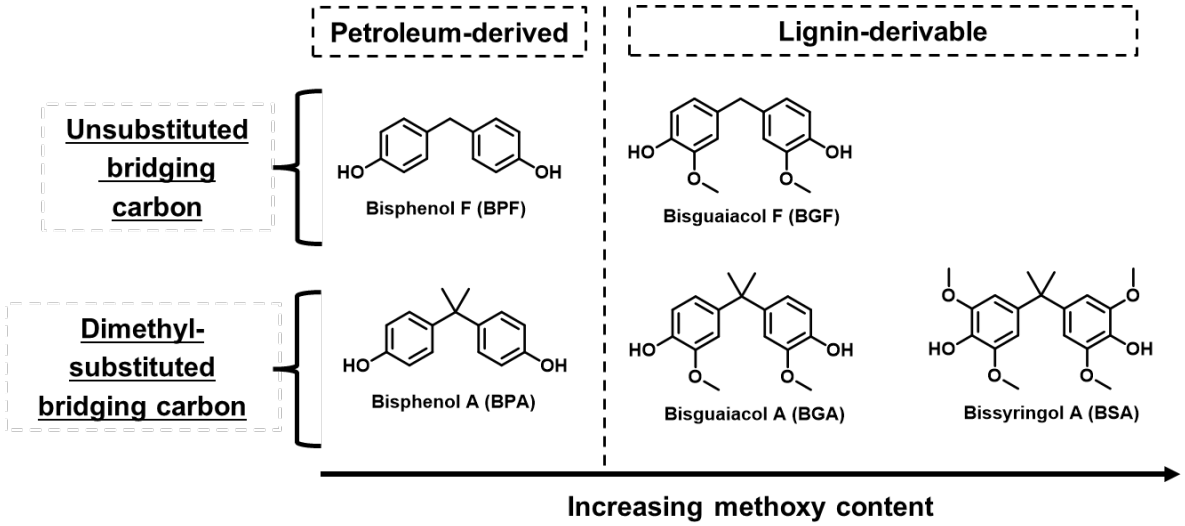

Figure 1: Structures of BPA, BPF, BGF, BGA, and BSA.

In this study, we examined two newly synthesized dimethyl-substituted, bridging carbon-based, lignin-derivable bisphenols (BGA, BSA) [structures shown in Figure 1] and investigated their possible EDEs and developmental toxicities. The toxicities of BGA and BSA were benchmarked against BPA to assess the potential of these lignin-derivable bisphenols as safer BPA replacements. We also included BGF and BPF in this work to compare the toxicity of unsubstituted, bridging carbon-based, lignin-derivable bisphenols with dimethyl-substituted versions. First, we applied two *in silico* methods – (i) structure-based molecular docking simulations to predict EDE

and (ii) quantitative structure–activity relationship (QSAR)-based toxicity estimation software tool (T.E.S.T.) to estimate developmental toxicity (Schneider et al., 2019). Second, we conducted an *in vitro* MCF-7 cell proliferation and *in vivo* chicken assays to evaluate the EA and developmental toxicity, respectively. Third, we measured the expression levels of two estrogen-induced chicken yolk proteins, vitellogenin II (VtgII) and very low-density apolipoprotein II (ApoII) by real-time polymerase chain reaction (rtPCR). The chicken yolk proteins are primarily generated in livers and respond to the circulating levels of estradiol, which makes chicken embryonic livers an excellent model for studying the EA of environmental contaminants (Evans et al., 1988). With this framework, we probed the structure-activity relationships of lignin-derivable bisphenols (BGA and BSA) to understand how the methoxy-group content and bridging-group substituents of these monomers impacts EA and developmental toxicity.

2. Materials and methods

2.1 Chemicals and supplies

BGA (≥99%), BSA (≥99%), and BGF (≥99%) were synthesized according to published literature (Epps, III et al., 2022; Nicastro et al., 2018). BPA (>99%) and BPF (≥99%) were purchased from TCI. 17β-estradiol (E2, ≥98%), dimethyl sulfoxide (DMSO, >99.7%), and phosphate-buffered saline (PBS, 1X) were purchased from Fisher Scientific (Waltham, MA). The MCF-7 human breast cancer cell line was purchased from the American Type Culture Collection (ATCC No. HTB-22). The Catalase Assay Kit (707002) and thiobarbituric acid reactive substance (TBARS) [TCA Method] Assay Kit (700870) used in the chicken embryonic assay were purchased from Cayman Chemical (MI, USA). The primers (β-actin, ApoII, and VTGII) and Gene Expression Master Mix were purchased from Integrated DNA Technologies, Inc (Coralville, IA). All chemicals were used as received without further purification.

2.2 Molecular docking

The endocrine disrupting potentials of E2, BPA, BPF, BGF, BGA, and BSA were assessed on the Docking Interface for Target Systems platform (named endocrine disruptome tool, http://endocrinedisruptome.ki.si) by AutoDock Vina (Kolšek et al., 2014). The ligand structures were generated by ChemSketch to obtain the SMILES (Simplified Molecular-Input Line Entry-System) files as inputs for the endocrine disruptome tool. The binding affinities were predicted

between ligands and 14 nuclear receptors, including androgen receptor (AR), estrogen receptors (ERs), glucocorticoid receptor (GR), liver X receptor (LXR), mineralocorticoid receptor (MR), peroxisome proliferator-activated receptor (PPAR), progesterone receptor (PR), retinoid X receptor (RXR), and thyroid receptor (TR). For androgen and estrogen receptors, both agonist and antagonist (an) conformations were included. All the information related to these protein receptors can be find on the endocrine disruptome tool website. Three thresholds were set per structure to divide test compounds into four probability binding classes (very strong binding, strong binding, moderate binding, and weak binding) (Kolšek et al., 2014).

2.3 T.E.S.T.

The acute toxicity (oral rat median lethal dose [LD_{50}]), developmental toxicity, and mutagenicity of the lignin-derivable monomers were predicted via T.E.S.T. (4.2.1) developed by the U.S. Environmental Protection Agency (EPA) (Martin, 2016). The structures shown in Figure 1 were generated using the structure drawing tool provided by the software. Once the toxicity endpoints (oral rat LD_{50}, developmental toxicity, and mutagenicity) and consensus method were chosen from the QSAR options, the analysis was executed, and a report with the predicted outcome was produced (Martin, 2016).

2.4 MCF-7 cell proliferation assay

The EA of lignin-derivable monomers was investigated using the MCF-7 cell proliferation assay with a concentration range from 10^{-12} to 10^{-7} M based on a procedure described in the literature (Peng et al., 2018; Zhang and Wu, 2022). E2 and BPA were included as the positive controls. Briefly, MCF-7 cells were seeded into 96-well plates containing EA-free culture medium and exposed to the test compounds for 6 days. Cell proliferation rates were measured via MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays by a microplate reader (Synergy^2, Bio-Tek, instruments, Winooski, VT). The EA of the test compounds was calculated as relative maximum %E2 (%RME2) = $100 \times (OD \text{ of test} - OD\ of\ VC)/(MAX\ OD\ of\ E2 - OD\ of\ VC)$.

145 ***2.5 Chicken embryonic assay***

146 **Experiment 1**

147 Fertilized Leghorn eggs (100) were obtained from the University of Delaware (UD)
148 research farm and were injected with the VC (0.1 vol% DMSO) and BPA at five dosages (0.2 mL
149 injection at 0.001 to 10 mM, resulting in final dosage at 0.76 to 7600 μg/kg) on day 6. Experiment
150 1 was conducted in three independent trials. The eggs were sealed with Duco Cement and returned
151 to incubation at 37 °C and 60% relative humidity. The eggs were candled every other day to assess
152 mortality. The incubation was terminated on day 18, and the embryos were dissected and assessed
153 for abnormality. Developmental indices, including embryo weight, liver somatic index (LSI), and
154 embryo-to-egg weight (REEW) ratio were recorded. LSI was calculated as liver mass/embryo
155 mass × 100%, and REEW was calculated as embryo mass/egg mass. The liver and brain tissues
156 were collected for TBARS level and catalase (CAT) activity measurements.

157 The TBARS level was measured on the tissue homogenates, following the protocol from the
158 TBARS (TCA Method) Assay Kit (Cayman Chemical, MI USA) and calculated as an index of
159 lipid peroxidation. Similarly, the liver and brain tissue homogenates were prepared using cold 50
160 mM potassium phosphate buffer (with 1 mM EDTA, pH 7.0), following the protocol from the
161 Catalase Assay Kit (Cayman Chemical, MI USA).

162

163 **Experiment 2**

164 Fertilized Leghorn eggs (136) were obtained from the UD research farm and were injected
165 with VC, E2, BPF, BGF, BGA, and BSA at two selected dosages (0.01 mM and 0.1 mM injection
166 concentration at 0.2 mL, resulting final dosage shown in Table 2) following the same procedure
167 as described in Experiment 1. Experiment 2 was conducted in two independent trials due to the
168 large group of test compounds. On day 18, all embryos were dissected, and the developmental
169 indexes were recorded and calculated as described above.

170

171 ***Ribonucleic acid (RNA) extraction and real time-PCR***

172 Eggs used for real time-PCR were injected with the test compounds at 0.1 mM (0.2 mL)
173 once per day on day 13 and day 15, which was the highest dosage used in the chicken embryonic
174 assay and also within the effective range for the target genes (Li et al., 2014). Livers from embryos
175 were collected on day 16, and total RNA was extracted from liver tissues using the RNeasy Plus

Mini Kit (QIAGEN, Germantown, MD) following the protocol from the manufacturer. The quality and concentration of RNA were determined using the NanoDrop One (Thermo Scientific). The RNA samples with the ratio of absorbance at 260 nm and 280 nm in the range of 1.9 – 2.2 were used for further steps. Total RNA (1 μg) was reverse transcribed by the QuantiTect Reverse Transcription Kit (QIAGEN, Germantown, MD). Then, real time-PCR was performed using complementary DNA (2 μL), 2X PrimTime gene expression master mix (10 μL, IDT), and a mixture of primers and probes (2 μL, shown in Table S1) on a BioRad CFX96 Real-Time System at a total reaction volume of 20 μL. We used β-actin as the housekeeping gene. All primer and probe sequences were listed in Table S1. The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.6 Log of (water solubility measured in mol/L) [log S] and water/octanol partition coefficient (log P) estimations

Log S and log P values were generated according to the methods described in the previous publication (Amitrano et al., 2021). Basically, the chemical structures of the tested bisphenols served as inputs to a web-based resource, Chemicalize.org by ChemAxon 2024 (https://chemaxon.com/calculators-and-predictors; accessed March 12, 2024). Then, the log S and log P values for the respective bisphenols were generated (ChemAxon, 2021).

2.7 Statistical analysis

For the MCF-7 cell proliferation assay, the calculated %RME2 values were analyzed using one-way analysis of variance (ANOVA) ($p < 0.05$) following Dunnett's method (comparison with the BPA group) in the statistical software package, JMP (JMP PRO 15) (Sall et al., 2017). The half-maximal effective concentration (EC_{50}) of the test compounds was determined by the statistical software GraphPad Prism 8 (Peng et al., 2018). For the chicken embryonic assay, the developmental indexes, CAT activity, TBARS values, and fold change of gene expression were evaluated by ANOVA ($p < 0.05$) following Dunnett's method (comparison with the VC group and/or BPA group). The viability data of chicken embryos were analyzed using Fisher's Exact Test in GraphPad Prism 8, and $p < 0.05$ was considered significant.

3. Results

3.1 In silico results of binding affinities and T.E.S.T. for E2, BPA, BPF, and three lignin-derivable monomers

The binding affinities of the test compounds (E2, BPA, BPF, BGF, BGA, and BSA) to 14 endocrine-related nuclear receptors were investigated using molecular docking; full data was shown in Table S2. As the positive control E2 showed high-level (very strong) binding affinities to AR (-10.5 kcal/mol), AR an (-10.1 kcal/mol), ER α (-10.6 kcal/mol), ER β (-10.0 kcal/mol), ER β an (-9.2 kcal/mol), and MR (-8.6 kcal/mol) [see Table S2]. Shown in Figure 2, the binding energies of test chemicals to 11 nuclear receptors exhibited differences between lignin-derivable monomers and the positive control. The dashed line/solid line shown in Figure 2 represented the threshold value for strong-binding ability of each receptor that is generated by the software. BGA had a comparable binding affinity among the majority of test receptors compared to BPA with exception of AR and MR, whereas BSA had much lower (weak) binding affinities to AR, ERs, MR, and TRs. Both BGF and BPF exhibited similarly weak binding to ERs and moderate binding affinities to TRs. However, BGF demonstrated lower binding affinities to GR and MR in comparison with BPF.

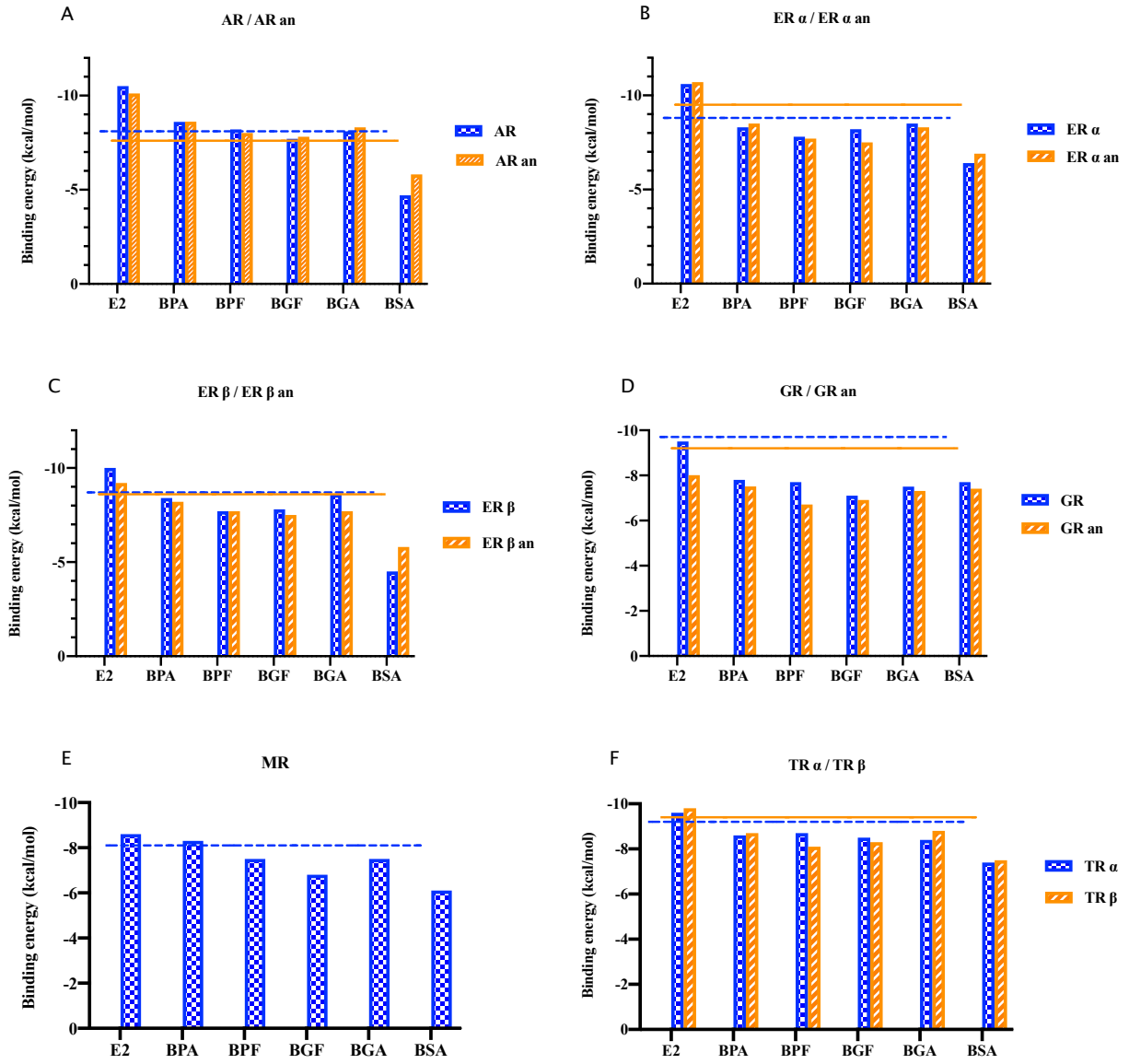

Figure 2: Binding energies (kcal/mol) of E2, BPA, BPF, BGF, BGA, and BSA to (A) AR and AR an, (B) ER α and ER α an, (C) ER β and ER β an, (D) GR and GR an, (E) MR, and (F) TR α and TR β. The dashed line/solid line at each figure represents the threshold value of the strong binding for the respective receptor. The threshold values were generated by the docking tool that separated the strong-binding group and moderate-binding group.

The predicted LD_{50} values (for rats), developmental toxicity, and mutagenicity for the test compounds from T.E.S.T. are shown in Table S3. All the test compounds were classified as non-mutagenic developmental toxicants with values higher than the threshold of 0.5. For the acute

toxicity to rats, the test chemicals had predicted LD_{50} values between 500 and 5000 mg/kg; thus, they were all classified in Category III (slightly toxic) on the basis of the EPA's 4-category hazard classification (Gadaleta et al., 2019). Category I ($LD_{50} \leq 50$ mg/kg) indicates the highest toxicity category, while Category IV ($LD_{50} > 5000$ mg/kg) indicates a safe chemical. It is important to note that this *in silico* toxicity prediction is part of the screening process, and an experimental method is employed for further assessing the developmental toxicity of test compounds.

3.2 EA evaluation of BPA, BPF, and lignin-derivable monomers by MCF-7 cell proliferation assay.

The potential EA of BPA, BPF, and three lignin-derivable monomers (BGA, BSA, BGF) was investigated using the MCF-7 cell proliferation assay at concentrations ranging from 10^{-12} M to 10^{-6} M (1 pM to 1 μM), which included the exposure levels of BPA in different countries (Forde et al., 2022; Li et al., 2023). In the current study, we applied %RME2 of VC + 3 SD (18%) as a cut-off value for detectable EA (shown in Figure 3, dashed line) (Yang et al., 2014). Results showed that E2 had detectable EA at all test concentrations (from 10^{-12} to 10^{-6} M) with the maximum EA value of 95.4% ± 11% at 10^{-9} M. BPA had detectable EA at five test concentrations from 10^{-10} M to 10^{-6} M with the highest EA value at 49.5% ± 18% (10^{-6} M). BGA had detectable EA at three out of seven concentrations (10^{-9}, 10^{-8}, and 10^{-6} M) with a higher maximum EA value of 47% ± 12% (at 10^{-8} M). Notably, BSA had undetectable EA (< 18%) at seven test concentrations. Next, BPF had the highest EA value of 42.6% ± 21% at 10^{-6} M, and it showed detectable EA from 10^{-8} M to 10^{-6} M. On the other hand, BGF showed detectable EA from 10^{-11} M to 10^{-9} M with a maximum EA of 28% ± 28% at 10^{-9} M. Compared with BPA, BGF had a significantly lower EA at 10^{-7} M ($p < 0.05$).

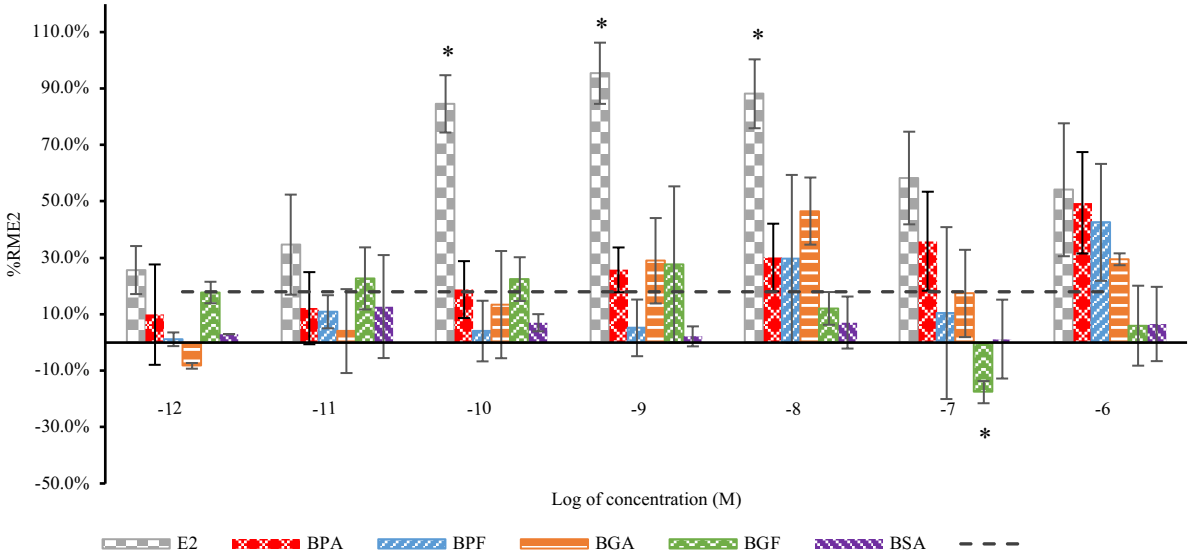

Figure 3: EA of E2, BPA, BPF, BGA, BGF, and BSA was quantified using the MCF-7 cell proliferation assay. E2 was a positive control, and %RME2 indicated the relative maximum %E2. A compound was considered to have no detectable EA when the %RME2 was lower than 18% (dashed line). The data were represented as mean ± SD of at least two independent trials run in triplicate. Differences are evaluated using one-way ANOVA followed by Dunnett's test in comparison to BPA. * indicates a significant difference between test compounds and BPA at the same concentration ($p < 0.05$).

3.3 Hepatic mRNA expression of the estrogen-responsive genes in chicken embryos

The hepatic mRNA expression levels of two estrogen-responsive genes (ApoII and VtgII) were assessed in the chicken embryos after exposure to E2, BPA, BPF, BGF, BGA, and BSA (at a non-toxic concentration of 0.1 mM). As shown in Figure 4, all treatments up-regulated ApoII gene expression in liver samples of day 16 embryos. E2, as the positive control, had a significantly higher fold change level (1198.51 ± 88.05) than other compounds. BPA had the highest fold change at 11.51 ± 3.36 among the other five treatments and was followed by BPF at 5.38 ± 2.45. Three lignin-derivable monomers had significantly lower expression levels (ranging from 1.84 to 4.41) of the ApoII gene in comparison to those of BPA and E2 ($p < 0.05$). On the other hand, except for the E2 group, the VtgII gene could not be consistently detected in other samples; thus, no fold change of gene expression was calculated (data not shown).

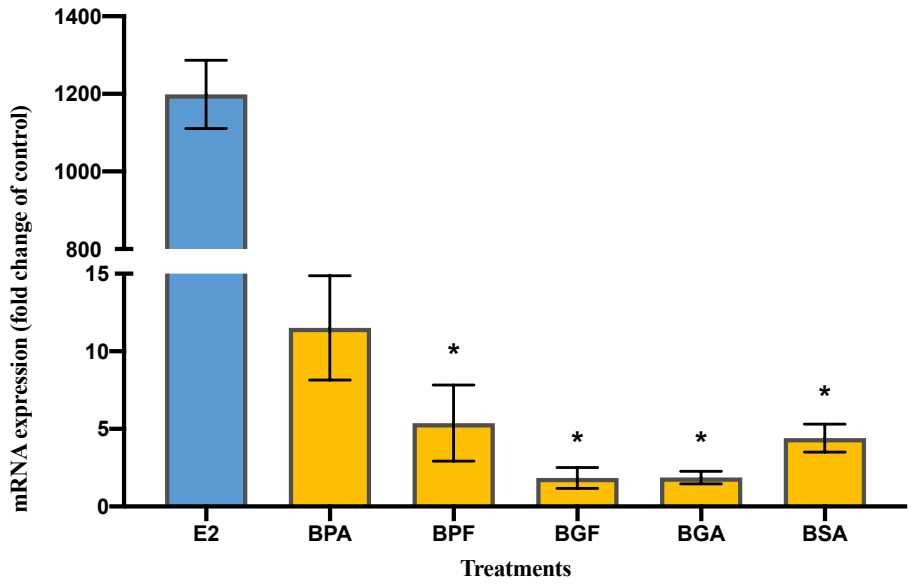

Figure 4: Effect of E2, BPA, BPF, and three lignin-derivable monomers on the mRNA expression of ApoII in the liver samples of day 16 female chicken embryo after injecting at day 13 and day 15 at 0.1 mM. Values are expressed as mean ± SD from three independent trials (n = 6), and significant changes are indicated relative to BPA (*$p < 0.05$; Dunnett's test).

3.4 Developmental toxicity assessment in chicken embryonic assay

Experiment 1: BPA treatment at five dosages

As shown in Table 1, highest exposure to BPA at 7600 µg/kg reduced the embryonic viability to 62.5% ($p < 0.05$), and the dosages at 76 µg/kg and 7.6 µg/kg both lowered the viability rate to 81.3%. The second highest dosage (760 µg/kg) and lowest dosage group (0.76 µg/kg) showed embryo viability of 94.8% and 87.5%, respectively. Additionally, deformed embryos (stunting, shown in Figure S2) were found in three BPA exposure groups at 0.76, 76, and 760 µg/kg. The REEW values were similar among five BPA treatments and the VC from 0.40 ± 0.03 to 0.42 ± 0.04, except for the highest BPA dosage group, which had a slightly lower number of 0.37 ± 0.05. The largest LSI% number was detected as 2.99% ± 0.35% at the dosage of 7600 µg/kg and the second largest as 2.98% ± 0.30% at the dosage of 0.76 µg/kg, but without significant difference from the VC (2.48% ± 0.28%) ($p > 0.05$). The liver weights showed a similar trend to the LSI% index after BPA exposure. However, due to the biological variance among the three trials, no significant difference was detected ($p > 0.05$).

Table 1: Effects of the BPA treatment at five exposure dosages on chicken embryonic viability, malformation status, REEW, LSI%, embryo weight, and liver weight of chicken embryos on day 18.

Treatment	Injection concen-tration (mM)	Final dosage (μg/kg egg)	Viability		Malformation		REEW	LSI (%)	Embryo weight (g)	Liver weight (g)
			Ratio	%	Ratio	%				
VC	0.1 vol% DMSO	NA	19/20	95	0/20	0	0.42 ± 0.04	2.48 ± 0.28	22.31 ± 1.90	0.57 ± 0.05
BPA	0.001	0.76	14/16	87.5	1/16	6.3	0.40 ± 0.03	2.98 ± 0.30	22.70 ± 2.29	0.67 ± 0.11
	0.01	7.6	13/16	81.3	0/16	0	0.41 ± 0.05	2.72 ± 0.21	21.77 ± 2.42	0.59 ± 0.03
	0.1	76	13/16	81.3	2/16	12.5	0.41 ± 0.05	2.60 ± 0.35	23.19 ± 1.04	0.60 ± 0.08
	1	760	15/16	94.8	2/16	12.5	0.40 ± 0.03	2.80 ± 0.30	21.69 ± 1.27	0.61 ± 0.06
	10	7600	10/16	62.5*	0/16	0	0.37 ± 0.05	2.99 ± 0.35	21.72 ± 1.25	0.65 ± 0.05

The data are presented as a total number of viability and malformation from three independent trials. Values of REEW, LSI (%), embryo weight, and liver weight are expressed as mean ± SD from three independent trials. The viability data were evaluated by Fisher's exact test, and * indicates a significant difference from the VC ($p < 0.05$).

Our results showed that the VC had the lowest TBARS values of 79.03 ± 4.41 nmol/g and 94.03 ± 14.13 nmol/g for liver and brain samples, respectively (shown in Figure S1 A and B). BPA treatment at the highest dosage (7600 μg/kg) significantly increased liver TBARS value to 101.75 ± 8.53 nmol/g ($p < 0.05$). Liver samples from the other four BPA dosages had increased TBARS values ranging from 88.69 ± 9.34 to 96.40 ± 9.90 nmol/g. However, only marginally elevated TBARS levels were found in the brain tissues after BPA exposure, ranging from 96.33 ± 22.17 to 105.30 ± 26.23 nmol/g without a statistically significant difference vs. the VC. Additionally, the VC had the highest CAT activity levels of 27.52 ± 6.54 and 0.31 ± 0.07 μmol/g for the liver and brain samples, respectively (shown in Figure S1C and D). In the liver samples, BPA treatment at five dosages decreased CAT activity values, ranging from 20.61 ± 2.38 to 22.96 ± 9.68 μmol/g ($p > 0.05$). A similar pattern of decreased CAT activity also was detected for brain tissues when

exposed to BPA, ranging from 0.25 ± 0.08 to 0.29 ± 0.08 μmol/g, without significant change in comparison to the VC.

Experiment 2: BPF, BGF, BGA, and BSA treatment at two dosages

Following the finding of BPA from Experiment 1, environmentally relevant exposure levels at injection concentrations 0.01 and 0.1 mM were applied in Experiment 2 to evaluate the developmental toxicity of three lignin-derivable monomers. E2 had a low viability rate of 70.6% (for 9.1 μg/kg) and 45.5% (91 μg/kg dosage) ($p < 0.05$). BGA and BSA showed viability rates of 75% and 87.5% at two test dosages that were comparable to that of BPA at 81.3%. On the other hand, BPF and BGF had the same viability rates of 100% (for low dosage) and 87.5% (for high dosage). Additionally, deformed embryos (*i.e.,* stunting and exposed brain) were detected in two E2 treatments at 11.8% and 9.1% for low and high dosages, respectively. The high-dosage BPA and the low-dosage BSA group also had a malformation rate of 12.5%. As shown in Table 2, BSA at the low dosage (11.6 μg/kg) had the lowest REEW value of 0.34 ± 0.03 but without significant difference ($p > 0.05$), which may be attributed to the stunted embryo detected in this group. The other groups had similar values ranging from 0.36 to 0.41. BPA, BPF, and three lignin-derivable monomers resulted in slightly higher LSI% values (2.56% - 2.77%) in comparison to the VC group of 2.31% ± 0.18% but with no statistical differences ($p > 0.05$).

Table 2: Effects of E2, BPA, BPF, BGA, BGF, and BSA treatments (at two injection concentrations) on chicken embryonic viability, malformation status, REEW, LSI (%), embryo weight, and liver weight of chicken embryos on day 18.

Treatment (molecular weight, g/mol)	Injection concent-ration (mM)	Final dosage (μg/kg egg)	Viability		Malformation		REEW	LSI (%)	Embryo weight (g)	Liver weight (g)
			Ratio	%	Ratio	%				
VC	0.1 vol% DMSO	NA	16/16	100	0/16	0	0.41 ± 0.01	2.31 ± 0.18	24.32 ± 0.71	0.56 ± 0.07
E2 (272.38)	0.01	9.1	24/34	70.6*	4/34	11.8	0.39 ± 0.00	2.18 ± 0.01	20.24 ± 3.53	0.54 ± 0.01
	0.1	91	16/22	45.5*	2/22	9.1	0.38 ± 0.02	2.06 ± 0.38	22.89 ± 0.87	0.47 ± 0.07
BPA (228.29)	0.01	7.6	13/16	81.3	0/16	0	0.41 ± 0.05	2.72 ± 0.21	21.77 ± 2.42	0.59 ± 0.03
	0.1	76	13/16	81.3	2/16	12.5	0.41 ± 0.05	2.60 ± 0.35	23.19 ± 1.04	0.60 ± 0.08
BPF (200.23)	0.01	6.7	8/8	100	0/8	0	0.36 ± 0.01	2.62 ± 0.12	22.10 ± 0.20	0.58 ± 0.03
	0.1	67	7/8	87.5	0/8	0	0.37 ± 0.00	2.56 ± 0.06	20.91 ± 0.12	0.54 ± 0.02
BGA (288.34)	0.01	9.6	7/8	87.5	0/8	0	0.40 ± 0.03	2.57 ± 0.01	22.92 ± 0.80	0.59 ± 0.02
	0.1	96	6/8	75	0/8	0	0.40 ± 0.03	2.73 ± 0.13	23.13 ± 0.38	0.64 ± 0.04
BGF (260.28)	0.01	8.7	8/8	100	0/8	0	0.40 ± 0.00	2.72 ± 0.10	23.33 ± 0.42	0.64 ± 0.01
	0.1	87	7/8	87.5	0/8	0	0.40 ± 0.03	2.69 ± 0.02	23.05 ± 0.78	0.62 ± 0.02
BSA (348.15)	0.01	11.6	7/8	87.5	1/8	12.5	0.34 ± 0.03	2.77 ± 0.21	20.40 ± 1.63	0.56 ± 0.00
	0.1	116	6/8	75	0/8	0	0.38 ± 0.01	2.63 ± 0.02	23.04 ± 0.86	0.62 ± 0.01

The data on viability and malformation are presented as a total number of inspections from two independent trials. Values of REEW, LSI (%), embryo weight, and liver weight are expressed as mean ± SD from two independent trials. Differences were evaluated using ANOVA followed by Dunnett's test. The viability data were evaluated by Fisher's exact test, and * indicates a significant difference relative to the VC ($p < 0.05$).

As shown in Figure S3, the VC had a TBARS value of 69.93 ± 8.60 nmol/g. E2 at the high dosage showed a significantly increased TBARS value of 99.20 ± 7.43 nmol/g vs. the VC ($p < 0.05$). At the low dosage treatment, E2 had TBARS level of 92.55 ± 3.39 nmol/g. BPA exposure raised the TBARS values to 90.25 ± 8.07 and 87.10 ± 14.36 for the low and high injection concentrations, respectively, but without a significant difference vs. the VC group. Additionally, at 0.01 mM BPF had a significantly decreased TBARS value vs. the BPA treatment ($p < 0.05$), whereas no statistically significant variations were found at other treatment groups vs. the BPA treatment.

361

362 ***4. Discussion***

363 ***4.1 EA of lignin-derivable monomers from in silico, in vitro, and in vivo assays***

364 We first applied molecular docking to the three lignin-derivable monomers to predict the
365 binding affinities of 14 nuclear receptors associated with the endocrine system. Results (Section
366 3.1) showed that BSA (with four methoxy groups, *i.e.,* two methoxy groups per aromatic ring) had
367 weaker binding affinities to the majority of receptors vs. BPA (with zero methoxy groups).
368 However, BPA and BGA with two methoxy groups (*i.e.,* one methoxy group per aromatic ring)
369 exhibited comparable binding affinities for most receptors (*e.g.*, ERs, GR, TRs). As one critical
370 pathway of EDCs, the EA of test compounds was further investigated experimentally via the MCF-
371 7 cell proliferation assay and fetal chicken hepatic mRNA expression of the estrogen-responsive
372 genes. Although the EA of BPA and bisphenol analogues (*e.g.,* BPF, BPAF, BPS) have been
373 widely studied in the MCF-7 cell model (Rivas et al., 2002), two-hybrid yeast bioassays (Lei et al.,
374 2017), and zebrafish-specific assays (Le Fol et al., 2017), much is unknown regarding the EA of
375 lignin-derivable monomers.

376

377 EA of the lignin-derivable monomers was assessed via MCF-7 cell proliferation assays at a
378 concentration range from 10^{-12} M to 10^{-6} M, equivalent to the BPA exposure levels in the U.S.
379 population as suggested by Peng et al. (2020). E2 (a natural estrogen hormone), as the positive
380 control, displayed a similar EA trend and EC_{50} value vs. those reported in a previous meta-analysis
381 result (Yang et al., 2014). BPA showed detectable EA at five test concentrations (10^{-10} M to 10^{-6}
382 M), whereas BPF had EA at 10^{-8} M and 10^{-6} M, but both had similar EC_{50}. This finding agreed
383 with a previous study that reported BPA with dimethyl substituents on the bridging carbon had
384 higher EA than BPF, which was related to hydrophobicity and rotational freedom (Maruyama et
385 al., 2013). BGA had the lowest EC_{50} value of 6.76×10^{-11} M and the second highest max %RME2
386 number of 47% among test compounds, whereas BSA displayed undetectable EA at all test
387 concentrations (10^{-12} M to 10^{-6} M). BGA with two methoxy groups (*i.e.,* one methoxy group per
388 aromatic ring) did not significantly lower EA in comparison to BPA. Thus, one methoxy group
389 per aromatic ring likely may not exhibit enough steric hinderance around the phenolic hydroxyl
390 group to limit access to the binding sites within ERs. However, with four methoxy groups (*i.e.,*
391 two methoxy groups per aromatic ring), BSA showed lower binding affinities to ERs in molecular

392 docking and undetectable EA in MCF-7 cells. Therefore, the two methoxy groups per aromatic
393 ring provide sufficient steric restrictions around the phenolic hydroxyl group to reduce the
394 interaction with binding sites. Together, the number of methoxy groups plays a critical role in
395 reducing EA of dimethyl-substituted bridging bisphenols. Notably, water solubility can be one of
396 several factors influencing the EA of bisphenols. The water solubility of a compound is related to
397 log S [log of (water solubility measured in mol/L)] value. We calculated the log S values for each
398 respective bisphenol [Table S5], and the results suggested that all bisphenols are slightly soluble
399 in an aqueous medium. As both environments (lipid/water) are present inside the biological body,
400 we also calculated the octanol-water partition coefficient (log P) values, which could more
401 accurately relate to EA. More positive log P values are associated with stronger
402 hydrophobicity/lipophilicity. It has been previously reported that the log P values of the
403 bisphenols decreased with the increased methoxy content, and this decreased
404 hydrophobicity/lipophilicity was in agreement with the weakening of the binding affinity of a
405 bisphenol to ERα (Amitrano et al., 2021). Our results are consistent with this previous work, as
406 BSA (with four methoxy groups) exhibited a lower log P value than that of BPA (with no methoxy
407 groups) and BGA (with two methoxy groups) [Table S5].
408
409 Additionally, we utilized a chicken embryo model targeting the expression of estrogenic-
410 responsive genes, serving as one of the biomarkers for EA testing of lignin-derivable monomers
411 at an environmentally relevant concentration (Li et al., 2014). As ovipara, the chicken liver is a
412 critical target organ for the steroid hormone estrogen, because it is the site for most yolk precursor
413 protein synthesis (Li et al., 2014). Notably, the injection and sample collection time play important
414 roles in the gene expression analysis of these two estrogen-responsive genes (VtgII and ApoII).
415 The capacity of these two genes to respond to estradiol fluctuated during the course of chicken
416 embryo development; it peaked in the mid-incubation time and declined in the late fetal
417 development stage (> embryonic day 17) (Evans et al., 1988). Thus, we chose the injection day at
418 the mid-incubation time (day 13 - 15) and collected the sample before the target gene expression
419 started to decline, which is day 16. Among two estrogen-dependent genes (VtgII and ApoII)
420 assessed, only the ApoII gene was successfully detected in all test groups, which agreed with
421 literature reports that the induction of ApoII mRNA would be a more sensitive endpoint than VtgII
422 (Lorenzen et al., 2003). Therefore, only the ApoII gene was subjected to gene expression analysis

in this study. Our results (Figure 4) showed that the E2 significantly stimulated ApoII expression with a roughly 1200-fold change vs. the control, which is consistent with an earlier study that E2 upregulated mRNA expression of ApoII in a dose-dependent pattern at 0.1 - 100 μg E2/egg (Li et al., 2014). Among the test substances, BPA exhibited the greatest effect on ApoII expression, with a fold change of 11.51 in comparison to the control. These results were in agreement with a study by Ma et al., in which BPA treatment increased ApoII mRNA levels in chicken embryonic hepatocytes in a concentration-dependent pattern (at 1 to 10 μM) (Ma et al., 2015). However, the three lignin-derivable compounds (BGA, BSA, and BGF) had significantly lower fold change values (at 1.84 to 5.38) of ApoII expression than BPA ($p < 0.05$), indicating a lack of estrogenic response in the chicken fetal liver. Notably, BGA showed a lower EA than BPA in the gene expression assay, but not in the molecular docking and MCF-7 cell proliferation assay. This discrepancy between *in vitro* and *in vivo* results can largely be attributed to the complexity of the *in vivo* models. The *in vitro* cellular studies are mainly impacted by the solubility and hydrophobicity of compounds. In contrast, the *in vivo* models are influenced by several additional factors such as the interactions between bisphenols and egg albumin (*i.e.,* environment) and distinct mechanisms from different *in vivo* systems. The bisguaiacols/bissyringols have at least one methoxy group ortho to the hydroxyl group on each aromatic ring that may increase steric hindrance around phenolic hydroxyls and thus reduce interactions with binding pockets on the estrogen receptors. As a result, the methoxy substituents significantly lowered the estrogenic-responsive gene expression level in comparison to BPA.

4.2 Developmental toxicity of lignin-derivable monomers from in silico simulation and chicken embryonic assay

In addition to the EA, developmental toxicity is another important toxicity endpoint related to EDCs. The developmental toxicity of BPA and BPF has been reported in various animal models, including rats (Lee et al., 2022), zebrafish (Gao et al., 2022), and chicken embryos (Mentor et al., 2020). To investigate the developmental toxicity of lignin-derivable monomers, we first applied T.E.S.T. to predict their acute toxicity and developmental toxicity (data shown in Table S3). Of note, even though the lignin-derivable monomers had varied oral rat LD_{50} values (~739 - 3196 mg/kg), they were assigned to Category III ($500 <$ oral rat $LD_{50} \leq 5000$ mg/kg; slightly toxic) according to the EPA's 4-category hazard classification (Gadaleta et al., 2019). Although the

454 QSARs-based simulations have been widely applied as valuable screening tools, it was still
455 challenging to predict the developmental toxicity thoroughly due to the lack of dose response and
456 its complex nature, as several organs and hormones were involved (Hulzebos et al., 2001).
457
458 Therefore, an *in vivo* chicken embryonic assay was applied to further investigate the
459 developmental toxicity of BPA and its potential alternatives. The chicken embryonic model has
460 been recently recognized as a promising alternative model to traditional rodent animals for
461 toxicological research (Ghimire et al., 2022). We first studied the developmental toxicity of BPA
462 at five dosages ranging from 0.76 to 7600 μg/kg (at injection concentrations 0.001 to 10 mM) to
463 cover the possible exposure level and explore the dose-response pattern. The results (Table 1)
464 revealed that BPA decreased the viability of chicken embryos with a non-monotonic dose response
465 (NMDR). We combined the viability data to perform statistical analysis (Fisher's exact test), which
466 is also the approach used in many toxicity studies employing the chicken embryo model (Szabó et
467 al., 2020; Hussein and Singh, 2016). Fisher's exact test is often used to analyze mortality data in
468 animal studies when dealing with categorical data, particularly in situations where sample sizes
469 are small. The highest mortality rate was detected at the highest dosage group (7600 BPA μg/kg
470 egg) and followed by two middle dosage groups (7.6 and 76 BPA μg/kg), whereas the second
471 highest dosage treatment (760 BPA μg/kg) had the lowest death rate. It has been widely reported
472 that NMDR was involved in various BPA toxicities, such as hormone-sensitive endpoints and
473 animal behavior studies (Yadav et al., 2022); however, the relevant mechanisms attributed to these
474 NMDR relationships are still not fully understood, especially for *in vivo* models (Lagarde et al.,
475 2015). Following the findings from BPA treatments, two intermediate and environmentally
476 relevant injection concentrations, 0.01 and 0.1 mM, were selected to determine the developmental
477 toxicity of lignin-derivable monomers (Dekant and Völkel, 2008). Except for the E2 groups
478 (positive control), there was no significant difference in viability rates between other treatments
479 and VC. BPF and BGF showed slightly higher viabilities (high dose: 87.5%; low dose: 100%) than
480 BPA, whereas BGA and BSA had a similar level of viability as BPA. These results also agreed
481 with the T.E.S.T. prediction that BPF and BGF had lower developmental toxicity values than BPA
482 and BGA. Although there was no available developmental toxicity data for BPF from chicken
483 embryos, previous studies showed that BPF had lower developmental toxicity than BPA in the
484 zebrafish embryo model regarding half-lethal concentrations and other developmental effects (Mu

et al., 2018; Gao et al., 2022). More studies in other animals are still warranted to further understand the developmental toxicities of these compounds.

To uncover the potential mechanism of oxidative stress on developmental toxicity, we monitored two crucial biomarkers for the antioxidant system in chicken embryos: TBARS and CAT activity. TBARS serves as an indicator of oxidative damage in tissue samples, reflecting the lipid peroxidation levels (Kourouma et al., 2015). CAT has been identified as the main hydrogen peroxide scavenger, and is one of the crucial enzymes in the endogenous antioxidant defense system (Haider et al., 2021). Our results (Figure S1) showed that the highest dosage of BPA exposure significantly increased TBARS level in liver samples ($p < 0.05$), whereas for the brain samples, only marginally increased TBARS levels were detected after BPA exposure ($p > 0.05$). Additionally, a declining trend in CAT enzyme activity was discovered in liver samples after BPA treatments, but no significant difference was found due to the large biological variability. The impaired antioxidant enzyme system and increased lipid peroxidation after BPA exposure have been reported in rat and mouse models (Meng et al., 2019; Kourouma et al., 2015). For example, Kourouma et al. reported that BPA exposure significantly elevated malondialdehyde (MDA) levels and lowered CAT activity in the livers of rats in a dose-dependent manner. The difference in the previous study is that they used a considerably larger exposure dosage range (at 2 - 50 mg/kg) and a longer period (30 days) in comparison to our study, which may have caused a noticeable increase in oxidative damage. Findings from CAT and TBARS assays indicated TBARS assay is a more sensitive assay for oxidative stress in response to low doses of BPA exposure at 0.76 - 7600 μg/kg. More data are needed to explore the longer-term exposure effects of these two indicators on both brain and liver tissues in the chicken embryo model.

Based on the results of Experiment 1, the liver was selected as the target organ for TBARS measurement, because it had a higher sensitivity than the brain samples. The significantly increased TBARS value ($p < 0.05$) was only detected in the higher dosage of the E2 group in comparison to the VC (shown in Figure S3). In the past decade, the chicken embryo model has already been used for toxicity evaluation of BPA. BPA treatment at 200 mg/L (on embryonic day 4) significantly increased the MDA value and reduced the glutathione level in the chicken embryo brain, impairing the antioxidant defense system. A recent study reported that BPA exposure at

0.228 and 2.28 mg/egg damaged neural tube development in a chicken embryo at an early stage (28 to 76 h of incubation) (Atay et al., 2020). Additionally, it has been reported that BPA at 48 mg/kg induced an estrogen-like effect in the chicken embryo by disrupting reproductive organ development (Mentor et al., 2020). However, all these studies applied a higher dosage range, more than 1000-fold higher than the possible human exposure level of BPA. In our study, we applied two exposure dosages of BPF and three lignin-derivable monomers from 6.7 to 116 μg/kg, which were much lower than the previous dose range of studies on BPA and were much closer to the average human intake, which is at 0.2 – 0.5 μg/kg body weight and can be up to 513.73 μg/person (Dekant and Völkel, 2008; Wang et al., 2020). Our chicken embryonic assay suggested that the three lignin-derivable monomers (BGF, BGA, and BSA), as well as BPA and BPF, did not induce a significantly higher mortality rate nor elevated TBARS levels at the test dosages. Oxidative stress is normally considered as a major toxicity mechanism, so low TBARS levels might be one potential underlying mechanism for the low developmental toxicity of these compounds.

Overall, our work highlighted the promising attributes of lignin-derivable alternatives to BPA, but it is important to acknowledge certain limitations. First, this work assessed the developmental toxicity of these compounds within a narrow range of environmentally relevant concentrations (0.01 and 0.1 mM). Subsequent research should be conducted on a broad dosage to comprehensively determine the dose-response relationship for comparing the NMDR effect. Second, this study focused on the toxicity profiles of individual lignin-derivable monomers. The combined impact of multiple lignin-derivable compounds, along with other environmental contaminants on estrogenic activity and developmental toxicity, may warrant further investigation. Third, the long-term toxicity effects and metabolic reactions of these lignin-derivable compounds (i.e., BSA and BGA) were not within the scope of this work, which remain important topics to ensure comprehensive safety assessments.

Conclusion

This work highlighted dimethyl-substituted, bridging carbon-based, lignin-derivable bisphenols (BGA and BSA) as potential replacements to BPA and evaluated their EA and developmental toxicity for the first time per our knowledge. Notably, BSA with four methoxy substituents exhibited significantly lower EA than BPA, with weaker binding affinities to ERs and

undetectable EA in the in vitro MCF-7 cell proliferation assay and in vivo chicken fetal liver test. BGA with two methoxy groups also showed limited EA. Importantly, no developmental toxicity was noted in chicken embryos at environmentally relevant doses for these bisphenols. These findings suggest that lignin-derivable monomers, especially BSA, are promising, safer alternatives to BPA and other commercial bisphenols.

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Disclaimer

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Abbreviations

An, antagonist conformation; ANOVA, analysis of variance; ApoII, apolipoprotein II; AR, androgen receptor; BGA, bisguaiacol A; BGF, bisguaiacol F; BGM, bisguaiacol M; BGP, bisguaiacol P; BGS, bisguaiacol S; BPA, bisphenol A; BPAF, bisphenol AF; BPF, bisphenol F; BPS, bisphenol S; BSA, bissyringol A; DMSO, dimethyl sulfoxide; DoTS, Docking Interface for Target Systems; EA, estrogenic activity; EC_{50}, half maximal effective concentration; EDCs, endocrine disrupting chemicals; EDE, endocrine disruption effect; EPA, Environmental Protection Agency; ER, estrogen receptor; E2, 17β-estradiol; FDA, Food and Drug Administration; GR,

glucocorticoid receptor; LD_{50}, median lethal dose; Log P, Octanol/water partition coefficient; Log S, water solubility measured in mol/L; LSI, liver somatic index; LXR, liver X receptor; MDA, malondialdehyde; MR, mineralocorticoid receptor; NIPUs, non-isocyanate polyurethanes; NMDR, non-monotonic dose response; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; PR, progesterone receptor; QSAR, Quantitative Structure–Activity Relationship; REEW, ratio of embryo to egg weight; %RME2, relative maximum %E2; RNA, ribonucleic acid; RXR, retinoid X receptor; TBARS, thiobarbituric acid reactive substance; T.E.S.T., Toxicity Estimation Software Tool; T_gs, glass transition temperatures; TR, thyroid receptor; UD, University of Delaware; VC, vehicle control; VtgII, vitellogenin II.

References

Amitrano, A., Mahajan, J.S., Korley, L.T.J., Epps, III, T.H., 2021. Estrogenic activity of lignin-derivable alternatives to bisphenol A assessed via molecular docking simulations. RSC Adv. 11, 22149–22158.

Atay, E., Ertekin, A., Bozkurt, E., Aslan, E., 2020. Impact of Bisphenol A on neural tube development in 48-hr chicken embryos. Birth Defects Res. 112, 1386–1396.

Bass, G.F., Epps, III, T.H., 2021. Recent developments towards performance-enhancing lignin-based polymers. Polym. Chem. 12, 4130–4158.

ChemAxon, 2021. ChemAxon–Software solutions and services for chemistry and biology.

Chen, J., Xiao, Y., Gai, Z., Li, R., Zhu, Z., Bai, C., Tanguay, R.L., Xu, X., Huang, C., Dong, Q., 2015. Reproductive toxicity of low level bisphenol A exposures in a two-generation zebrafish assay: evidence of male-specific effects. Aquat. Toxicol. 169, 204–214.

Cywar, R.M., Rorrer, N.A., Hoyt, C.B., Beckham, G.T., Chen, E.Y.-X., 2022. Bio-based polymers with performance-advantaged properties. Nat. Rev. Mater. 7, 83–103.

Dekant, W., Völkel, W., 2008. Human exposure to bisphenol A by biomonitoring: methods, results and assessment of environmental exposures. Toxicol. Appl. Pharmacol. 228, 114–134.

Ďurovcová, I., Kyzek, S., Fabová, J., Makuková, J., Gálová, E., Ševčovičová, A., 2022. Genotoxic potential of bisphenol A: A review. Environ. Pollut. 306, 119346.

Epps, III, T.H., Korley, L.T.J., Green, M.D., Mahajan, J.S., Hoda, S.B., 2022. Bio-based polysulfones and uses thereof, US Pat. application,.

Erler, C., Novak, J., 2010. Bisphenol a exposure: human risk and health policy. J. Pediatr. Nurs. 25, 400–407.

613 Evans, M.I., Silva, R., Burch, J.B., 1988. Isolation of chicken vitellogenin I and III cDNAs and
614 the developmental regulation of five estrogen-responsive genes in the embryonic liver.
615 Genes Dev. 2, 116–124.

616 Forde, M.S., Côté, S., Laouan Sidi, E.A., Gaudreau, É., Ayotte, P., 2022. Evaluation of
617 Bisphenol A in Pregnant Women from 10 Caribbean Countries. Toxics 10, 556.

618 Gadaleta, D., Vuković, K., Toma, C., Lavado, G.J., Karmaus, A.L., Mansouri, K., Kleinstreuer,
619 N.C., Benfenati, E., Roncaglioni, A., 2019. SAR and QSAR modeling of a large collection
620 of LD50 rat acute oral toxicity data. J. Cheminform. 11, 1–16.

621 Gao, Y., Li, A., Zhang, W., Pang, S., Liang, Y., Song, M., 2022. Assessing the toxicity of
622 bisphenol A and its six alternatives on zebrafish embryo/larvae. Aquat. Toxicol. 246,
623 106154.

624 Ghimire, S., Zhang, X., Zhang, J., Wu, C., 2022. Use of Chicken Embryo Model in Toxicity
625 Studies of Endocrine-Disrupting Chemicals and Nanoparticles. Chem. Res. Toxicol. 35,
626 550–568.

627 Haider, M.S., Jaskani, M.J., Fang, J., 2021. Overproduction of ROS: underlying molecular
628 mechanism of scavenging and redox signaling, in: Biocontrol Agents and Secondary
629 Metabolites. Elsevier, pp. 347–382.

630 Harnett, K.G., Moore, L.G., Chin, A., Cohen, I.C., Lautrup, R.R., Schuh, S.M., 2021.
631 Teratogenicity and toxicity of the new BPA alternative TMBPF, and BPA, BPS, and BPAF
632 in chick embryonic development. Curr. Res. Toxicol. 2, 399–410.

633 Hulzebos, E.M., Janssen, P.A.H., Maslankiewicz, L., Meijerink, M.C.M., Muller, J.J.A.,
634 Pelgrom, S.M.G., Verdam, L., Vermeire, T.G., 2001. The application of structure-activity
635 relationships in human hazard assessment: a first approach.

636 Hussein, M., Singh, V., 2016. Effect on chick embryos development after exposure to
637 neonicotinoid insecticide imidacloprid. J. Anat. Soc. India 65, 83–89.

638 Kolšek, K., Mavri, J., Sollner Dolenc, M., Gobec, S., Turk, S., 2014. Endocrine disruptome - An
639 open source prediction tool for assessing endocrine disruption potential through nuclear
640 receptor binding. J. Chem. Inf. Model. 54, 1254–1267. https://doi.org/10.1021/ci400649p

641 Kourouma, A., Quan, C., Duan, P., Qi, S., Yu, T., Wang, Y., Yang, K., 2015. Bisphenol A
642 induces apoptosis in liver cells through induction of ROS. Adv. Toxicol. 2015.

643 Lagarde, F., Beausoleil, C., Belcher, S.M., Belzunces, L.P., Emond, C., Guerbet, M., Rousselle,
644 C., 2015. Non-monotonic dose-response relationships and endocrine disruptors: a
645 qualitative method of assessment. Environ. Heal. 14, 1–15.

646 Le Fol, V., Aït-Aïssa, S., Sonavane, M., Porcher, J.-M., Balaguer, P., Cravedi, J.-P., Zalko, D.,
647 Brion, F., 2017. In vitro and in vivo estrogenic activity of BPA, BPF and BPS in zebrafish-
648 specific assays. Ecotoxicol. Environ. Saf. 142, 150–156.

649 Lee, S.-J., Baek, S.-K., Kim, W., Quah, Y., Kim, S.-Y., Jeong, J.-S., Lee, J., Yu, W.-J., 2022.
650 Reproductive and developmental toxicity screening of bisphenol F by oral gavage in rats.
651 Regul. Toxicol. Pharmacol. 136, 105286.

652 Lei, B., Xu, J., Peng, W., Wen, Y., Zeng, X., Yu, Z., Wang, Y., Chen, T., 2017. In vitro profiling
653 of toxicity and endocrine disrupting effects of bisphenol analogues by employing MCF-7
654 cells and two-hybrid yeast bioassay. Environ. Toxicol. 32, 278–289.

655 Li, J., Leghari, I.H., He, B., Zeng, W., Mi, Y., Zhang, C., 2014. Estrogen stimulates expression
656 of chicken hepatic vitellogenin II and very low-density apolipoprotein II through ER-α.
657 Theriogenology 82, 517–524.

658 Li, R., Zhan, W., Ren, J., Zhang, F., Huang, X., Ma, Y., 2023. Temporal trends in risk of
659 bisphenol A, benzophenone-3 and triclosan exposure among US children and adolescents
660 aged 6–19 years: Findings from the National Health and Nutrition Examination Survey
661 2005–2016. Environ. Res. 216, 114474.

662 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time
663 quantitative PCR and the 2− ΔΔCT method. methods 25, 402–408.

664 Lorenzen, A., Williams, K.L., Oon, T.W.M., 2003. Determination of the estrogenic and
665 antiestrogenic effects of environmental contaminants in chicken embryo hepatocyte cultures
666 by quantitative-polymerase chain reaction. Environ. Toxicol. Chem. An Int. J. 22, 2329–
667 2336.

668 Ma, M., Crump, D., Farmahin, R., Kennedy, S.W., 2015. Comparing the effects of
669 tetrabromobisphenol-A, bisphenol A, and their potential replacement alternatives, TBBPA-
670 bis(2,3-dibromopropyl ether) and bisphenol S, on cell viability and messenger ribonucleic
671 acid expression in chicken embryonic hepatocytes. Environ. Toxicol. Chem. 34, 391–401.
672 https://doi.org/10.1002/etc.2814

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

676 Martin, T., 2016. User's guide for TEST (version 4.2)(Toxicity Estimation Software Tool) A
677 program to estimate toxicity from molecular structure. US EPA Office of Research and
678 Development. EPA/600/R-16/058.

679 Maruyama, K., Nakamura, M., Tomoshige, S., Sugita, K., Makishima, M., Hashimoto, Y.,
680 Ishikawa, M., 2013. Structure–activity relationships of bisphenol A analogs at estrogen
681 receptors (ERs): Discovery of an ERα-selective antagonist. Bioorg. Med. Chem. Lett. 23,
682 4031–4036.

683 Meng, Z., Tian, S., Yan, J., Jia, M., Yan, S., Li, R., Zhang, R., Zhu, W., Zhou, Z., 2019. Effects
684 of perinatal exposure to BPA, BPF and BPAF on liver function in male mouse offspring
685 involving in oxidative damage and metabolic disorder. Environ. Pollut. 247, 935–943.

686 Mentor, A., Wänn, M., Brunström, B., Jönsson, M., Mattsson, A., 2020. Bisphenol AF and
687 bisphenol F induce similar feminizing effects in chicken embryo testis as bisphenol A.
688 Toxicol. Sci. 178, 239–250.

689 Mhatre, S. V, Mahajan, J.S., Epps, III, T.H., Korley, L.T.J., 2023. Lignin-derivable alternatives
690 to petroleum-derived non-isocyanate polyurethane thermosets with enhanced toughness.
691 Mater. Adv. 4, 110–121.

692 Moreman, J., Lee, O., Trznadel, M., David, A., Kudoh, T., Tyler, C.R., 2017. Acute toxicity,
693 teratogenic, and estrogenic effects of bisphenol A and its alternative replacements bisphenol
694 S, bisphenol F, and bisphenol AF in zebrafish embryo-larvae. Environ. Sci. Technol. 51,
695 12796–12805.

696 Mu, X., Huang, Y., Li, Xuxing, Lei, Y., Teng, M., Li, Xuefeng, Wang, C., Li, Y., 2018.
697 Developmental effects and estrogenicity of bisphenol A alternatives in a zebrafish embryo
698 model. Environ. Sci. Technol. 52, 3222–3231.

699 Nicastro, K.H., Kloxin, C.J., Epps, III, T.H., 2018. Potential lignin-derived alternatives to
700 bisphenol a in diamine-hardened epoxy resins. ACS Sustain. Chem. Eng. 6, 14812–14819.

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

704 Peng, Y., Nicastro, K.H., Epps, III, T.H., Wu, C., 2020. Methoxy groups reduced the estrogenic
705 activity of lignin-derivable replacements relative to bisphenol A and bisphenol F as studied
706 through two in vitro assays. Food Chem. 338, 127656.

707 Peng, Y., Nicastro, K.H., Epps, III, T.H., Wu, C., 2018. Evaluation of estrogenic activity of
708 novel bisphenol A alternatives, four bioinspired bisguaiacol F specimens, by in vitro assays.
709 J. Agric. Food Chem. 66, 11775–11783.

710 Rivas, A., Lacroix, M., Olea-Serrano, F., Laïos, I., Leclercq, G., Olea, N., 2002. Estrogenic
711 effect of a series of bisphenol analogues on gene and protein expression in MCF-7 breast
712 cancer cells. J. Steroid Biochem. Mol. Biol. 82, 45–53.

713 Sall, J., Stephens, M.L., Lehman, A., Loring, S., 2017. JMP start statistics: a guide to statistics
714 and data analysis using JMP. Sas Institute.

715 Schneider, M., Pons, J.L., Labesse, G., Bourguet, W., 2019. In silico predictions of endocrine
716 disruptors properties. Endocrinol. (United States) 160, 2709–2716.
717 https://doi.org/10.1210/en.2019-00382

718 Schutyser, W., Renders, ab T., Van den Bosch, S., Koelewijn, S.-F., Beckham, G.T., Sels, B.F.,
719 2018. Chemicals from lignin: an interplay of lignocellulose fractionation, depolymerisation,
720 and upgrading. Chem. Soc. Rev. 47, 852–908.

721 SZABÓ, R., CSONKA, D., MAJOR, L., LEHEL, J., BUDAI, P., 2020. Toxicity test of
722 individual and combined toxic effects of glyphosate herbicide and heavy metals on chicken
723 embryos. AGROFOR 5.

724 Trullemans, L., Koelewijn, S.-F., Scodeller, I., Hendrickx, T., Van Puyvelde, P., Sels, B.F.,
725 2021. A guide towards safe, functional and renewable BPA alternatives by rational
726 molecular design: structure–property and structure–toxicity relationships. Polym. Chem. 12,
727 5870–5901.

728 Usman, A., Ahmad, M., 2019. Computational study suggesting reconsideration of BPA
729 analogues based on their endocrine disrupting potential estimated by binding affinities to
730 nuclear receptors. Ecotoxicol. Environ. Saf. 171, 154–161.
731 https://doi.org/10.1016/j.ecoenv.2018.12.071

Vom Saal, F.S., Nagel, S.C., Coe, B.L., Angle, B.M., Taylor, J.A., 2012. The estrogenic endocrine disrupting chemical bisphenol A (BPA) and obesity. Mol. Cell. Endocrinol. 354, 74–84.

Wang, H., Liu, Z., Zhang, J., Huang, R.-P., Yin, H., Dang, Z., 2020. Human exposure of bisphenol A and its analogues: understandings from human urinary excretion data and wastewater-based epidemiology. Environ. Sci. Pollut. Res. 27, 3247–3256.

Yadav, S.K., Bijalwan, V., Yadav, S., Sarkar, K., Das, S., Singh, D.P., 2022. Susceptibility of male reproductive system to bisphenol A, an endocrine disruptor: Updates from epidemiological and experimental evidence. J. Biochem. Mol. Toxicol. e23292.

Yang, C.Z., Casey, W., Stoner, M.A., Kollessery, G.J., Wong, A.W., Bittner, G.D., 2014. A Robotic MCF-7: WS8 cell proliferation assay to detect agonist and antagonist estrogenic activity. Toxicol. Sci. 137, 335–349. https://doi.org/10.1093/toxsci/kft250

Yin, L., Dai, Y., Cui, Z., Jiang, X., Liu, W., Han, F., Lin, A., Cao, J., Liu, J., 2017. The regulation of cellular apoptosis by the ROS-triggered PERK/EIF2α/chop pathway plays a vital role in bisphenol A-induced male reproductive toxicity. Toxicol. Appl. Pharmacol. 314, 98–108.

Yin, N., Liang, X., Liang, Shengxian, Liang, Shaojun, Yang, R., Hu, B., Cheng, Z., Liu, Shuyu, Dong, H., Liu, Sijin, 2019. Embryonic stem cell-and transcriptomics-based in vitro analyses reveal that bisphenols A, F and S have similar and very complex potential developmental toxicities. Ecotoxicol. Environ. Saf. 176, 330–338.

Zhang, X., Mahajan, J.S., Korley, L.T.J., Epps, III, T.H., Wu, C., 2022. Reduced genotoxicity of lignin-derivable replacements to bisphenol A studied using in silico, in vitro, and in vivo methods. Mutat. Res. Toxicol. Environ. Mutagen. 503577.

Zhang, X., Wu, C., 2022. In Silico, In Vitro, and In Vivo Evaluation of the Developmental Toxicity, Estrogenic Activity, and Mutagenicity of Four Natural Phenolic Flavonoids at Low Exposure Levels. ACS omega 7, 4757–4768.