

The impact of differential lignin S/G ratios on mutagenicity and chicken embryonic toxicity

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3 **Abstract**
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6 3 Lignin and lignin-based materials have received considerable attention in various fields due to
7 4 their promise as sustainable feedstocks. Guaiacol (G) and syringol (S) are two primary
8 5 monolignols that occur in different ratios for different plant species. As methoxyphenols, G and S
9 6 have been targeted as atmospheric pollutants and their acute toxicity examined. However, there
10 7 is a rare understanding of the toxicological properties on other endpoints and mixture effects of
11 8 these monolignols. To fill this knowledge gap, our study investigated the impact of different S/G
12 9 ratios (0.5, 1, and 2) and three lignin depolymerization samples from poplar, pine, and
13 10 miscanthus species on mutagenicity and developmental toxicity. A multi-tiered method
14 11 consisted with *in silico* simulation, *in vitro* Ames test, and *in vivo* chicken embryonic assay was
15 12 employed. In the Ames test, syringol showed a sign of mutagenicity, while guaiacol did not,
16 13 which agreed with the T.E.S.T. simulation. For three S and G mixture and lignin monomers,
17 14 mutagenic activity was related to the proportion of syringol. In addition, both S and G showed
18 15 developmental toxicity in the chicken embryonic assay and T.E.S.T. simulation, and guaiacol
19 16 had a severe effect on lipid peroxidation. A similar trend and comparable developmental toxicity
20 17 levels were detected for S and G mixtures and the three lignin depolymerized monomers. This
21 18 study provides data and insights on the differential toxicity of varying S/G ratios for some
22 19 important building blocks for bio-based materials.
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26 **Short abstract**
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29 23 To understand the toxicity impacts of S/G ratios from various lignocellulosic biomass
30 24 feedstocks, we used a multi-tiered platform to study the toxicities of the pure S and G, mixtures
31 25 at different S/G ratios, and three lignin depolymerization samples. In the Ames test, syringol
32 26 showed a sign of mutagenicity while guaiacol did not, which agreed with the T.E.S.T. The S/G
33 27 mixtures and lignin samples revealed the importance of the S/G ratio on mutagenicity in the
34 28 Ames test, and developmental toxicity in the chicken embryos.
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37 **Key words**
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39 32 Guaiacol; syringol; lignin; developmental toxicity; mutagenicity; *in silico* simulation
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1. Introduction

Lignocellulosic biomass (LB) is an abundant resource existing in plants, which has great potential as an alternative feedstock for fuels and chemicals (Brethauer & Studer, 2015). Three major components of LB are cellulose, hemicelluloses, and lignin, which are naturally recalcitrant to microbial and enzymatic degradation. The composition of lignocellulosic biomass varies depending on species and their sources, such as hardwoods, softwoods, and grasses. Cellulose is the most abundant LB polymer, representing 40–60% of the biomass weight, consisting of D-glucose subunits linked by β -1,4 glycosidic bonds (Pérez, Munoz-Dorado, De la Rubia, & Martinez, 2002). Hemicelluloses are complex heterogeneous carbohydrates, consisting of a mixture of monosaccharide subunits: pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and sugar acids (4-O-methyl-glucuronic, galacturonic and glucuronic acids) (Khalaf, 2016). Lignin is the second most abundant natural polymer on earth after cellulose. It comprises around 30% of the mass of softwoods and 20-25% in hardwood trees (Sen, Patil, & Argyropoulos, 2015). As a primary structural component of cell walls, lignin is essential to plants, providing mechanical support, aiding in the transport water and nutrients, and protecting them from microbial attack (Sen et al., 2015).

Lignin, a phenylpropanoid polymer, is biosynthesized in plants from the polymerization of the three precursors of p-hydroxycinnamyl alcohols: coniferyl alcohol (CA), sinapyl alcohol (SA), and p-coumaryl alcohol. Respectively, they produce the guaiacyl (G), syringyl (S), and hydroxyphenyl (H) residues in natural polymers (Rodrigues, Meier, Faix, & Pereira, 1999). The component proportion and structure of lignin varies depending on the plant species and environmental factors. In hardwoods, lignin consists of S units and G units (guaiacyl-syringyl lignin), while the softwood lignin mainly consists of only G units (more than 95%), and grass lignin consists of G, S, and H units (Fukushima, 2001). These units are linked by ether and carbon-carbon bonds repeated in an irregular form, such as alkyl-aryl ether linkages (β -O-4), β -5, β - β , 4-O-5, and 5-5 linkages. Among them, the β -O-4 is the most abundant lignin linkage and is considered to be the only one with an uncondensed structure. A correlation between the β -O-4 structure and the S/G ratio has been detected, which indicates that the syringyl/guaiacyl composition affects the proportion of erythro and threo forms of β -O-4 structure in hardwood lignin (R. B. Santos, Capanema, Balakshin, Chang, & Jameel, 2012). Compared to the softwood lignin, a greater variance of lignin structure among different species and a higher level of erythro form has been revealed for hardwood lignin (Kishimoto et al., 2010).

The S/G ratio has been determined for a variety of tree species using analytical pyrolysis. For example, the S/G ratio of *Eucalyptus globulus* wood (*E. globulus*) ranged from 1.64 to 2.32 (Alves et al., 2011), whereas the S/G ratio from lignin in a series of natural poplar variants (genus *Populus*) ranged from 1.41 to 3.60 (Anderson et al., 2019). The S/G ratio is important because it is associated with the pulping yields since the S lignin has higher reactivity than G in alkaline systems (José et al., 2005). Furthermore, lignin, with a variety of aromatic groups, shows promise as a bio-feedstock (Nikafshar et al., 2017). Basically, due to its chemical structure and a number of hydrogen bonds, lignin possesses a very interesting thermal behavior and behaves as a thermoplastic (Jeong et al., 2013; Laurichesse and Avérous, 2014). Besides the thermal and mechanical properties, safety is another crucial factor for bio-synthesized polymers. The aromatic compounds, such as syringol and guaiacol, can be used to synthesize thermoplastic polymers using different polymerization methods applied in various areas (Llevot, Grau, Carlotti,

80 Grelier, & Cramail, 2016). Syringol has a similar structure to guaiacol, with one additional
81 methoxy group. Due to the different chemical structure, the toxicity profiles of syringol and
82 guaiacol are different. Acute oral toxicity test is the most fundamental and common test in
83 toxicology, which can be used for chemical hazard classification. Traditionally, the acute oral
84 toxicity data is obtained from different animal species (such as mice and rat, although only the
85 rat is used for classification purposes) and expressed as the lethal dosage that kills 50% of the
86 population (LD₅₀) of animals tested (Russo et al., 2019).

88 The acute toxicity values of rat LD₅₀ for guaiacol and syringol are 520 mg/kg and 550 mg/kg,
89 respectively (Orłowski & Boruszak, 1991). However, other toxicology aspects of these
90 significant lignin components are less understood except for the acute toxicity. Given increasing
91 production levels of lignin-based biopolymers (Kai et al., 2016), it is inevitable that humans will
92 be exposed to them at some level. Therefore, as the vital building block units for bio-based
93 materials, it is crucial to understand a wider range of toxicity of syringol and guaiacol and their
94 mixture with different S/G ratios. In addition, humans can be exposed to them from smoked
95 foods at 0.5–1.7 mg/kg and up to 18.4 mg/kg for some heavily smoked foods (Clifford, 2000).
96 Currently, there is a knowledge gap regarding the full range of toxicology profiles of lignin
97 components of bio-based materials, as well as the relationship between their monomer
98 composition and toxicity reaction. This study builds on the existing literature by using a multi-
99 tiered approach to investigate the two essential toxicity aspects (mutagenicity and developmental
100 toxicity) of S, G, and their mixture with varying proportions. Specifically, we evaluate the
101 mutagenic and developmental toxicity of S, G, and the mixture of different S/G ratios (S/G= 0.5,
102 1, and 2) using a platform combined *in silico*, *in vitro*, and *in vivo* models as shown in Figure 1.
103 We included the *in silico* simulation (Toxicity Estimation Software Tool; T.E.S.T.) as the first
104 step for toxicity evaluation due to its cost-effectiveness and efficiency. The mutagenic activity
105 and developmental toxicity were further assessed using the Ames test (at 0.001 to 1 mM) and
106 chicken embryonic assay (at 41.3 to 513 µg/kg), respectively. These test dosages were chosen
107 based on potential exposure level for bisphenol A and other fossil fuel-based polymer materials,
108 since syringol and guaiacol were good building blocks for bio-based acrylates and polymer
109 production (Erler and Novak, 2010; Veith et al., 2020). Moreover, we applied the toxicity
110 assessment for three lignin monomers isolated from miscanthus, poplar, and pine with different
111 S/G ratios (from 0.067 to 0.85). Thus, this study will contribute to our knowledge on the
112 different toxicity endpoints of varying S/G ratios by the multi-tiered approaches. Additionally,
113 besides the pure S and G mixtures, three plant samples with different S/G ratios have been
114 included, which will extend our knowledge on the toxicology profiles of lignin-based
115 biopolymers with different S/G ratios.

117 **2. Methods**

119 **2.1 Chemicals and materials**

121 All biomass samples were obtained from the Idaho National Laboratory and fully characterized
122 using the NREL LAP protocols. The samples were milled to particles ranging from 0.42 mm (40
123 mesh) – 2 (10 mesh) mm by Forest Concepts and used as received. 5 wt.% Ru/C powder was
124 purchased from Sigma Aldrich and used as received. Methanol (certified ACS Reagent Grade,
125 99.8%) was purchased from Fisher Chemicals and used as received. DMSO-d₆, pyridine-d₅ were
126 purchased from Sigma Aldrich. Deionized water (Millipore model Direct Q3 UV R) was used for

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3 127 all preparations requiring water. 17 β -estradiol (E2), dimethyl sulfoxide (DMSO) (D1391), and
4 128 phosphate buffered solution (PBS) (Gibco, 20-012-027) were purchased from Fisher Scientific
5 129 (Waltham, MA, USA). The three *Salmonella typhimurium* tester strains (TA 98, TA 100, and TA
6 130 102), top agar, Oxoid Nutrient Broth No.2, S9 mixture solutions were purchased from Molecular
7 131 Toxicology Inc (Boone, NC, USA).
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9 133 The molecular weight (MW) for syringol and guaiacol were 154.16 and 124.14 g/mol. The
10 134 mixtures of S and G at three different ratios (0.5, 1, and 2) were prepared by mixing the S and G
11 135 ratio mole at 1 M and then diluted to the final dose. The average MW for three S/G mixtures was
12 136 134.15, 139.15, and 144.15 g/mol at S/G=0.5, 1, and 2. The average MW for three plant lignin
13 137 monomers are as below based on the genus: pine (*Pinus* spp.): 140 g/mol, miscanthus (MC,
14 138 *Miscanthus* spp): 140 g/mol, poplar (*Populus* spp.): 146 g/mol.
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16 140 **2.2 Preparation and characterization of lignin monomer samples**

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18 142 **Reductive catalytic fractionation (RCF) of plant lignin.** Sample of 40 g of biomass
19 143 (poplar, pine and miscanthus) was added to 800 ml methanol in a 1.2 L high-pressure Parr reactor
20 144 along with 4 g Ru/C. The reactor was stirred with a mechanical stirrer and heated with a high-
21 145 temperature heating jacket connected to a variable power supply controlled by a PID temperature
22 146 controller and a K-type thermocouple to measure the reaction temperature through a thermowell.
23 147 Once sealed, the reactor was purged three times with N₂ and then pressurized with 40 bars of H₂.
24 148 The reactor was heated to 250 °C (it takes ~10 – 15 min to reach the set point) and held for 15 h
25 149 while stirring. Reaction conditions were optimized in our previous studies (Ebikade et al., 2020;
26 150 Li et al., 2018; Shuai et al., 2018; S. Wang, Shuai, Saha, Vlachos, & Epps, 2018). Subsequently,
27 151 the reactor was cooled until reaching room temperature and the gas phase was released. A portion
28 152 of the reaction products was filtered for monomer identification and quantification. The remaining
29 153 liquid was filtrated through a nylon membrane filter (Whatman®, 0.2 μ m) and the filtrate was
30 154 stored for further analyses.
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32 156 **Isolation of lignin monomers.** After evaporating methanol from the lignin product solution, 10 ml
33 157 of cyclohexane (to remove monomers from the lignin oil) was added to the viscous lignin oil. The
34 158 mixture was vortexed for 30 seconds and placed in a sonicating bath for 1 hour. The cyclohexane
35 159 layer was collected for monomer recovery and fresh cyclohexane was added, vortexed and
36 160 sonicated for two more monomer removal steps. After the three-time cyclohexane extraction, the
37 161 monomers were recovered following evaporation of cyclohexane.
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39 163 **Nuclear magnetic resonance.** Heteronuclear single quantum coherence (HSQC) nuclear
40 164 magnetic resonance (NMR) spectra of extracted lignin oils and isolated oligomer oils were
41 165 recorded at 25 °C on an Avance III 400 MHz NMR spectrometer (Bruker). Approximately 30 mg
42 166 of filtered lignin oil was dissolved in 500 μ l of premixed DMSO-d₆/pyridine-d₅ (4:1) prepared in
43 167 quartz NMR tubes (NewEra). Data processing was performed using the Mestrelab Research
44 168 software (mNOVA).
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46 170 **2.3 Toxicity Estimation Software Tool (T.E.S.T.)**

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3 172 The Toxicity Estimation Software Tool (T.E.S.T.) was developed by United States
4 173 Environmental Protection Agency (EPA) and used a variety of QSAR methodologies, including
5 174 Hierarchical, FDA, Single model, Group contribution, Nearest neighbor method, to estimate
6 175 toxicity and physical properties of test chemicals (Toxicity Estimation Software Tool (TEST)
7 176 2016). The predicted toxicity data presented in this study was generated from the Consensus
8 177 method which was the average of the predicted toxicities of previous five QSAR methods. The
9 178 endpoints included in our study: Oral rat LD 50, Developmental Toxicity, and Mutagenicity.
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12 180 **2.4 Ames test**

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14 182 The Ames test was conducted by three *Salmonella typhimurium* tester strains (TA 98, TA 100,
15 183 and TA 102) using a preincubated method as described by Maron & Ames (1983). The TA98
16 184 and TA100 strains were suggested as viable alternatives to the current OECD Test Guideline
17 185 TG471 by several recent studies (Williams et al., 2019; Gao et al., 2021; Khan et al., 2021). We
18 186 also included the TA102 as an additional test strain in our modified approach. The strains were
19 187 grown overnight in Oxoid Nutrient Broth No.2 and incubated in a shaking incubator at 37°C and
20 188 100 rpm to reach cell densities at $1-2 \times 10^9$ cells/mL. Each strain was conducted both in the
21 189 absence and in the presence of a metabolic activation mixture S9. S9 was freshly prepared before
22 190 each test by addition of liver extracts of Sprague–Dawley rats induced with Aroclor 1254,
23 191 regensys "A" and "B". The PBS was used as an S9 alternative for the test without S9 activation.
24 192 Each test compound was firstly dissolved in DMSO and then diluted by PBS buffer to reach the
25 193 concentration at 0.01 to 1 mM. The 0.05 ml of tested compounds were added to a 0.5 mL of S9
26 194 mixture (or 0.5 mL PBS in without S9 mixture). Then 0.1 mL of three bacterial culture added to
27 195 the mixture and incubated at 37 °C. After 30 min incubation, the 2 mL of top agar was added to
28 196 each tube and mixed well. The mixture was poured on to a minimal agar plate. The three
29 197 treatment dosages from 1 to 0.01 mM at 0.05 mL addition in each plate yielded the final
30 198 concentration from 50 to 0.5 nmol/plate. After 48 h incubation, the His⁺ revertant colonies on
31 199 plates were counted manually. Each test was repeated in two independent trials and duplicate for
32 200 each trial.
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34 202 **2.5 Chicken embryonic assay**

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36 204 **Egg treatments.** A total of 160 fertilized Leghorn eggs were obtained from the University of
37 205 Delaware research farm and used in the assay. The chicken embryo as an *in vivo* model using
38 206 early life stages have been widely used in toxicity assessment (Uggini, Patel, & Balakrishnan,
39 207 2012; Mentor, Bornehag, Jönsson, & Mattsson, 2020) The eggs were weighed and divided into
40 208 17 groups: vehicle control, and two dosages of guaiacol, syringol, three S/G mixtures (at ratio
41 209 0.5, 1, and 2), and three plant lignin monomers (MC, pine, and poplar). The chicken embryos on
42 210 day 6 were injected with each chemical solution (1 mM and 0.1 mM) or 1% DMSO in PBS
43 211 (vehicle control) at 0.2 ml using a syringe (1 ml). The final doses in egg (average of 60 g per
44 212 egg) included: 513 µg syringol/kg, 51.3 µg syringol/kg, 413 µg guaiacol/kg, 41.3 µg guaiacol/kg,
45 213 467 µg pine/kg, 46.7 µg pine/kg, 467 µg MC/kg, 46.7 µg MC/kg, 487 µg poplar/kg, and 48.7 µg
46 214 poplar/kg. After the treatments, eggs were put back to the egg incubator at 38 °C and 60%
47 215 relative humidity.
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217 **Developmental toxicity evaluation.** After chemical treatments, the eggs were candled every two
218 days and recorded for dead embryo numbers. The embryos were euthanized on day 18 by placing
219 them in the refrigerator at 4°C overnight. After eggs were opened, all embryos were weighted
220 and recorded for abnormality. Additional measurements were conducted on liver mass and heart
221 mass after embryos were dissected. The liver somatic index (LSI) was calculated as $LSI = \text{liver}$
222 $\text{mass}/\text{embryo mass} \times 100\%$, which reflected the health indicator after embryo exposure to the
223 environmental contaminant. The significantly changed values of LSI ($p < 0.05$) were identified
224 after comparison to the solvent control group, which indicates health problems in the chicken
225 embryo development (Guo et al. 2018; Mentor, Wänn, et al. 2020).

227 The liver samples from each treatment were collected for lipid peroxidation measurement. The
228 liver samples were firstly placed on ice and homogenized with buffer to get liver tissue
229 homogenates. The liver oxidative stress level was measured by quantifying the malondialdehyde
230 (MDA) level in fetal liver tissue homogenates. The TBARS Assay Kit (Cayman Chemical, MI
231 USA) was applied for the MDA assessment. Briefly, MDA reacted with thiobarbituric acid
232 (TBA) under acidic conditions and high temperature (around 100 °C) to form an MDA-TBA
233 adduct. The MDA-TBA adduct was measured colorimetrically at 530 nm and compared with the
234 values obtained from MDA standards. Results were expressed as nmol MDA/g liver
235 homogenates. Each test was repeated twice independently and in duplicate for each trial.

237 2.6 Data analysis

239 The results were analyzed with the statistical software package JMP (JMP PRO 15). In the
240 chicken embryonic assay, the morphological, developmental endpoints among groups, and lipid
241 oxidation level were all determined using a one-way analysis of variance (ANOVA) followed by
242 the Tukey's test for multiple comparisons between control and each treatment. Changes were
243 considered statistically significant if $p < 0.05$. In the Ames test, the data (revertants/plate) was
244 assessed by one-way analysis of variance (ANOVA) followed by the Tukey's test for multiple
245 comparisons between control and each treatment. The mutagenic index (MI) was also calculated
246 for each concentration using the mean number of revertants per plate with the test compound
247 divided by the mean number of revertants per plate with the negative (solvent) control. When
248 determining "mutagenicity", a tested compound was regarded as mutagenic if a two-fold
249 increase in the number of mutants ($MI \geq 2$) was detected in at least one concentration (Resende,
250 Vilegas, Dos Santos, & Varanda, 2012). For the "sign of mutagenicity", the compound that
251 didn't reach the two-fold increase but showed statistical significance ($p < 0.05$) of revertant
252 number as compared to the negative control was defined as having a sign of mutagenicity.

253 3. Results

256 3.1. Guaiacol had distinguished toxicity profile when compared with the findings of 257 syringol by *in silico* simulation

258 The acute toxicity (oral rat LD50), developmental toxicity, and mutagenicity were simulated by
259 the T.E.S.T. using the consensus method. The acute toxicity for the chemical classification was
260 based on the oral rat LD50. When the value was between 300 to 2000, the chemical belongs to
261 class 4 (class 1-5 with class 1 represents the most severe toxicity). As shown in Table 1, guaiacol
262 showed higher developmental toxicity and higher acute toxicity (lower oral rat LD 50 value) than

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3 263 syringol. For the mutagenicity, syringol had a higher value at 0.55 than guaiacol (0.11) and was
4 264 classified as mutagenicity positive. On the other hand, guaiacol was regarded as mutagenicity
5 265 negative. Interestingly, with an additional CH₃O group in syringol, it has higher mutagenicity but
6 266 lowers developmental toxicity.
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8 268 **3.2 Different mutagenicity was related with three S/G ratios and three different lignin** 9 269 **monomers**

10 270 The mutagenic activity of guaiacol, syringol, three S/G mixtures (at ratio 0.5, 1, and 2), and three
11 271 lignin monomers (MC, pine, and poplar) was evaluated by the Ames test at three concentrations
12 272 (0.5, 5, and 50 nmol/plate). As shown in Table 2 and 3, the positive control of each bacterial
13 273 strain (TA 98, TA 100, and TA 102) with or without S9, produced statistically significant
14 274 increases in the number of revertant colonies, and negative controls of three strains were in our
15 275 historical ranges of number of revertant colonies, which confirmed the sensitivity and accuracy
16 276 of the test system. Syringol significantly increased revertant number of TA 98 and TA102 strains
17 277 at 5 and 50 nmol/plate, with MI at 1.3 ($p < 0.05$). In contrast, there was no significant increased
18 278 number of revertant colonies for guaiacol treatments. The experimental findings agreed quite
19 279 well with the results from the *in silico* method, T.E.S.T. simulation, as summarized above (Table
20 280 1). Among the three S/G mixtures, the S/G ratio at 1 and 2 significantly increased revertant
21 281 numbers of TA 102 strain without S9 activation ($p < 0.05$), while the mixture with S/G ratio at
22 282 0.5 had no significant increase. Additionally, the mixture with a higher S/G ratio (S/G=2) at 50
23 283 nmol/plate had a higher number of revertant colonies than the mixture with the lowest S/G at 0.5
24 284 at the same concentration for TA 102 strain (at 50 nmol/plate, $p < 0.05$). With MI <2, the
25 285 increase in the sign of mutagenicity of three mixtures was largely associated with the bigger
26 286 content of syringol in the mixtures.
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28 288 As shown in Table 3, the mutagenic activity of three lignin monomers varied in the Ames test,
29 289 but the higher sign of mutagenicity was still recorded with larger S/G ratios. The poplar lignin
30 290 monomers, with the highest S/G ratio among these three samples, showed the highest MI values,
31 291 up to 1.8 at TA 98 strain. After TA 98 strain without S9 activation was treated with the poplar
32 292 lignin monomer at 0.5 to 50 nmol/plate, the MI levels were between 1.6 to 1.8. When MIs of the
33 293 TA 98 strain (with and without S9 activation) were compared between the treatments of pine and
34 294 poplar lignin monomers at 50 nmol/plate, a significantly smaller value of MI was detected in the
35 295 pine lignin monomer treatment ($p < 0.05$). Similar findings were also determined for the TA 102
36 296 revertants when treated with the pine samples at 50 nmol/plate ($p < 0.05$).
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38 298 **3.3 Different S/G ratios impacted differently the chicken embryonic and developmental** 39 299 **toxicity**

40 300 Table 4 summarizes the mortality and malformation number of chicken embryos after exposure
41 301 to S, G, three S/G mixtures, and three lignin monomers at two injection concentrations (0.1 and 1
42 302 mM), which yielded different final dose in eggs due the difference in molecular weight. The
43 303 fertilized eggs were randomly assigned to each treatment on day 6. Four more fertilized egg were
44 304 recorded in three mixture groups at high dose injection (1 mM) because one more trial (4 eggs)
45 305 was included to confirm the findings. One out of eight embryos was dead after each guaiacol
46 306 treatment, and 25% stunting embryos were detected in the 413 µg/kg guaiacol group, while only
47 307 one death was found for both syringol groups. Higher developmental toxicity was determined for
48 308 guaiacol which is in good agreement with the T.E.S.T simulation for both chemicals. The
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3 309 T.E.S.T. results showed that the syringol and guaiacol were both developmental toxicants, and
4 310 guaiacol showed a higher toxicity value than the findings of syringol.
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6 312 There were no clear associations between S/G ratios (or the mixture concentration) and the
7 313 chicken developmental toxicities. The 12.5% death rates were both recorded for the 0.5 and 2
8 314 S/G ratio mixtures groups at the lower dosage, while 8.3% and 25% were determined for the
9 315 higher dose respectively. Interestingly, the lowest death rates were determined for the middle
10 316 ratio mixture (S/G=1) at 0 and 8.3% for 46.3 and 463 $\mu\text{g}/\text{kg}$, respectively. After exposure to the
11 317 three lignin monomer samples, different chicken mortality was determined for each plant lignin
12 318 monomer sample, with the highest death at 25% after exposure to the higher dose of pine (467
13 319 $\mu\text{g}/\text{kg}$) and the lower dose of miscanthus (46.7 $\mu\text{g}/\text{kg}$) lignin monomers. The pine lignin with the
14 320 smallest S/G ratio at 0.067 indicated the highest proportion of guaiacol. Guaiacol had higher
15 321 developmental toxicity than the findings in syringol, as shown in Tables 1 and 4, which might
16 322 explain the higher developmental toxicity in the pine samples compared with the findings from
17 323 the other two plant samples.
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19 325 As shown in Table 5, the ratio of embryo weight to egg weight (REEW) decreased after
20 326 exposure to higher dose of guaiacol (413 $\mu\text{g}/\text{kg}$) and lower dose of S/G=0.5 mixture (44.6 $\mu\text{g}/\text{kg}$)
21 327 ($p < 0.05$). The LSI was calculated for each group and served as a general indicator of health
22 328 response from exposure to an environmental contaminant. The higher dose of guaiacol (413
23 329 $\mu\text{g}/\text{kg}$) decreased the LSI values significantly more than the solvent control ($p < 0.05$). Smaller
24 330 REEW also resulted in significantly lower LSI values. The three lignin monomer mixtures had
25 331 similar REEW and LSI. Additionally, the liver weight for each treatment also reflected the toxic
26 332 impacts from the same chemical treatments. Exposure to guaiacol (413 $\mu\text{g}/\text{kg}$) and the mixture at
27 333 S/G=0.5 (44.6 $\mu\text{g}/\text{kg}$) resulted in significantly decreased liver weights ($p < 0.05$) compared to the
28 334 value in the solvent control group, and there was no difference among the chicken heart weights
29 335 from all the treatments. The chicken liver was the first organ to respond to containments and
30 336 might be impacted more than chicken hearts.
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32 338 The MDA levels for each treatment group were determined to evaluate the oxidative stress level
33 339 of fetal chicken livers (Fig. 2). The solvent control (1% DMSO) showed the lowest MDA level at
34 340 45.01 ± 6.35 . Guaiacol and the S/G=0.5 mixture at the lower concentration significantly
35 341 increased the MDA level than the MDA value in the control group, at 91.57 ± 14.38 and 102.52
36 342 ± 3.93 , respectively ($p < 0.05$). The higher MDA values indicated more oxidative stress,
37 343 contributing to lower REEW and LSI in the lower dose of S/G mixture (S/G=0.5). Moreover,
38 344 among the three lignin monomers, a significantly increased MDA value was detected in the pine
39 345 group, which has the lowest S/G ratio at 0.067 ($p < 0.05$). With guaiacol having higher
40 346 developmental toxicity and MDA values than the findings in syringol (Table 1, 4, and 5, Fig. 2),
41 347 pine samples showed the highest MDA when compared with the findings from the other two plant
42 348 samples with larger S/G ratios. The greater oxidative stress in the treatment of the pine samples
43 349 could be associated with the highest chicken embryonic death (Table 4) among all 3 test plant
44 350 lignin monomers.
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4. Discussion

4.1 Differential toxicity profiles between guaiacol and syringol by *in silico* simulation and *in vitro* and *in vivo* experiments

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3 355 Besides serving as precursors of plastic polymer, the methoxyphenols (MPs) were regarded as
4 356 atmosphere pollutants generated from lignin pyrolysis during biomass burning (Collard & Blin,
5 357 2014). The 2-methoxyphenol (guaiacol), 2, 6-dimethoxyphenol (syringol), along with their
6 358 derivatives, were prominent types of MPs existing in lignin. As biomarkers for woodsmoke
7 359 exposure, both of them have been detected in human urine at concentrations of $8 \mu\text{g}/\text{m}^3$ (Dills et
8 360 al., 2006). However, there was little knowledge about the toxicity of guaiacol and syringol,
9 361 except their basic acute toxicity. Guaiacol has been regarded as harmful for aquatic organisms,
10 362 which belong to the 'harmful' and 'slightly toxic' hazard classes using luminescence test
11 363 according to the European and American legislation, respectively (Pflieger & Kroflič, 2017).
12 364 Furthermore, belong to the MPs, syringol had one more methoxy group than guaiacol, which
13 365 attributed to their potential different toxicology profiles. One previous study revealed that
14 366 methoxyphenols with a shorter alkyl chain showed weaker aquatic toxicity than longer alkyl
15 367 chains. In specific, compared with guaiacol, two 4-substituted guaiacols, creosol (4-
16 368 methylguaiacol) and 4-ethylguaiacol (4-EG) had higher toxicity on green algae, daphnia, and fish
17 369 (Wei et al., 2018). Therefore, in this study, we investigated two critical toxicity endpoints:
18 370 mutagenic activity and the developmental toxicity of guaiacol, syringol, and three mixtures.
19 371 Additionally, we extended the assessment to lignin monomers isolated from three test species
20 372 (poplar, pine, and miscanthus).
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22 374 In T.E.S.T. simulation, the guaiacol had higher acute toxicity than syringol, and both of them
23 375 were classified in Acute toxicity class 4. The oral rat LD50 value of guaiacol was 468.73 mg/kg
24 376 and 755.83 mg/kg for syringol (Table 1). Compared with the existing experimental oral rat LD50
25 377 for guaiacol at 520 mg/kg and syringol at 550 mg/kg (Orłowski & Boruszak, 1991), the oral rat
26 378 LD50 of guaiacol from T.E.S.T had even lower value than reported experimental data while
27 379 syringol had higher values than the experimental values. In addition, in the rat test, guaiacol only
28 380 produced slightly higher acute toxicity than that from syringol, while the difference of oral rat
29 381 LD50 between guaiacol and syringol was bigger from the T.E.S.T. simulation. Regarding the
30 382 mutagenicity, syringol showed a higher mutagenicity value and belonged to the mutagenicity
31 383 positive, while guaiacol belonged to the mutagenicity negative. The finding of higher potential
32 384 mutagenicity of syringol than guaiacol from *in vitro* Ames test was consistent with the simulated
33 385 mutagenicity results. In the Ames test, the revertant numbers of each solvent control were within
34 386 the historical ranges and control limits of our laboratory and agreed with values reported in the
35 387 literature (Levy et al., 2019). We applied the mean ± 2 standard deviations as our control limits
36 388 suggested by Kato et al. (2018). The higher MI values (up to 1.3) were detected after exposure to
37 389 syringol than guaiacol at TA 98 and TA 102 strains (5 and 50 nmol/plate). The mutagenicity of
38 390 syringol and guaiacol at higher concentrations (30 $\mu\text{mol}/\text{plate}$) was evaluated in a previous study
39 391 as tobacco smoke constituents and no mutagenic activity was reported (Florin, Rutberg, Curvall,
40 392 & Enzell, 1980). Regarding the developmental toxicity, T.E.S.T finding showed that guaiacol
41 393 had a higher developmental toxicity value (0.71) than syringol (0.54), and they all belonged to
42 394 the developmental toxicants. To confirm the simulation results, we applied a chicken embryo
43 395 model for assessment, which serves as a promising alternative method to traditional animal
44 396 studies (Samak et al., 2020). In this study, the adverse effects of chicken embryogenesis were
45 397 observed after exposure to both guaiacol and syringol at two doses. The 12.5% death rates were
46 398 detected for two doses of guaiacol (41.3 and 413 $\mu\text{g}/\text{kg}$) and the lower dose of syringol (51.3
47 399 $\mu\text{g}/\text{kg}$) groups. Additionally, 25% of deformed embryos (stunting) were found after exposure to
48 400 the 413 μg guaiacol/kg. Due to the different MW between guaiacol (124.14 g/mol) and syringol
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3 401 (154.16 g/mol), they had different final doses in egg even from the same injection concentrations
4 402 (0.1 and 1 mM). Higher doses per egg in $\mu\text{g}/\text{kg}$ were observed in syringol than in guaiacol, but
5 403 guaiacol still showed higher mortality as well as higher deformation rate at higher dose group.
6 404 Additionally, the higher mortality rate was observed in the lower dose of syringol group, which
7 405 indicated the presence of non-monotonic dose response (NMDR) effects (Vandenberg et al.,
8 406 2012). The NMDR included different shape of dose-response curves than the traditional one,
9 407 which means that the lower dose might have a larger effect than that of the higher dose.
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11 409 **4.2 Mutagenicity increased with higher S/G ratio among three different S/G ratios and** 12 410 **three different lignin monomers**

13 411 Lignin-based polymer materials have been reported increasingly applied as an alternative green
14 412 materials (Kai et al., 2016; C. Wang, Kelley, & Venditti, 2016). However, the toxicology data
15 413 was limited to single hazard components present in the lignin, and the assessment of a set of
16 414 toxicity properties was absent, which includes different aspects (acute toxicity, mutagenicity, and
17 415 developmental toxicity). Poplar, pine and miscanthus are promising lignocellulosic feedstocks on
18 416 the production of biofuels and biomaterials (Sannigrahi, Ragauskas, & Tuskan, 2010). As a
19 417 phenolic polymer, lignin is composed principally of three alcohols (p-coumaryl, coniferyl, and
20 418 sinapyl) and generated as different structural units (H, G, and S) by polymerization (A. C. dos
21 419 Santos, Ximenes, Kim, & Ladisch, 2019). The prooxidant activity and toxic effect also exist in
22 420 plant phenolic compounds, such as flavonoids and lignin precursors (Sakihama, Cohen, Grace, &
23 421 Yamasaki, 2002). In this study, we extended the mutagenicity assessment to three S/G mixtures
24 422 (from 0.5 to 2) and three lignin monomers (pine, miscanthus, and poplar) at different S/G ratios
25 423 (from 0.067 to 0.85). We specifically focused on relatively low exposure levels (at 0.001 to 1
26 424 mM) that may be easily overlooked but are nonetheless important for evaluation since they could
27 425 be related to human potential exposure levels with potential NMDR behavior and low dose
28 426 effects. The NMDR behavior of genotoxicity has been reported using *in vivo* comet assay in a
29 427 zebrafish embryo model for freshwater sediment samples due to overlapping cytotoxic effects
30 428 (Garcia-Käuffer et al., 2015). In addition, the antimicrobial effects of the three lignin
31 429 depolymerized monomers were detected. The minimal inhibitory concentrations against *S. aureus*
32 430 and *E. coli* were at 2.5 mg/mL and thus limited the high test concentrations in the Ames tests. In
33 431 this study, the highest and significantly different MI values indicating signs of mutagenicity were
34 432 observed at middle doses for syringol and S/G=1 mixture in TA 102 strain, with S9 activation
35 433 and without S9, respectively. In agreement with pure S and G data, the mixtures and lignin
36 434 samples with higher S ratios showed a higher mutagenic index. Significantly increased revertant
37 435 numbers were detected for S/G equal to 2 and 1 treatments ($p < 0.05$), but not in the group with
38 436 lower S/G value (S/G=0.5). For the three lignin monomers, a higher MI value up to 1.8 was
39 437 observed after exposure to poplar (S/G=0.85) at 50 nmol/plate for the TA 98 strain, showing a
40 438 significant sign of mutagenicity. Furthermore, compared with poplar treatments, the pine with
41 439 the lowest S/G ratio showed significantly reduced revertant numbers for the TA 98 and TA 102
42 440 strains. Because TA 98 and TA 1537 detect frame shifts, while TA 100 and TA 1535 detect
43 441 mutagens which cause base-pair substitutions, and TA 102 detects transition mutagens
44 442 containing nucleotides AT (Vijay et al. 2018), it is possible to just use the three strains— TA 98,
45 443 TA 100, and TA 102— in this study for the examination of frame shifts, base-pair substitutions
46 444 and transition mutagens containing nucleotides AT, respectively. Williams et al (2019)
47 445 suggested that test strains TA98 and TA100 were enough for detecting most bacterial mutagens
48 446 (93%); while including an *in vitro* assay that detects clastogens, such as the *in vitro* chromosome
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3 447 aberration assay, would detect 99% of bacterial mutagens. With the addition of TA 102 in our
4 448 current approach, we could determine higher bacterial mutagens than that suggested by Williams
5 449 et al (2019). This study is only the first stage for toxicity evaluation of these biomass-based
6 450 materials using a multi-tiered platform including *in silico* simulation, *in vitro* Ames test, and *in*
7 451 *vivo* chicken embryonic assay. In this study we target most bacterial mutagens. For our future
8 452 extensive mutagenicity studies, we will use the recommendation from the updated OECD TG
9 453 471(OECD, 2020) for the compounds or mixture showing high toxicity risks, and we fully
10 454 understand the benefits of using the five strains as they can test the wide possibility of
11 455 mutagenicity. In addition, we will include other *in vivo* mutagenicity tests, such as Comet assay,
12 456 to assess the genotoxicity more comprehensively and detect potential NMDR in a wider dosage
13 457 range.
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18 460 **4.3 Varying S/G ratios differentially impacted the chicken embryonic and developmental** 19 461 **toxicity**

20 462 Furthermore, the developmental toxicity of phenolic compounds has been reported in different
21 463 models (Yang et al., 2018; Chao et al., 2020). In our results, syringol and guaiacol showed
22 464 different toxicity profiles on the two endpoints: mutagenicity and developmental toxicity. The
23 465 adverse effects on chicken embryonic development were observed in both S and G compounds.
24 466 The three S/G mixtures groups all increased embryo mortality, with the highest death rate
25 467 observed at the higher dose of S/G=2 group at 25%. Additionally, the significant alteration of
26 468 several developmental indexes, including small REEW and liver weight, was detected at the
27 469 lower dose of the S/G=0.5 mixture. Moreover, a significantly increased MDA level, as a
28 470 biomarker of lipid peroxidation, in the liver sample was observed in the same group compared to
29 471 the control (Kurantowicz et al., 2017). The one potential mechanism of adverse effects on
30 472 embryonic development was related to oxidative stress damage(Nguyen et al., 2020). For the
31 473 S/G=2 treatments, the high mortality and malformation rates did not exist with alternated other
32 474 developmental indexes or increased MDA level, which suggested that other mechanisms might
33 475 play roles, such as anti-angiogenic or apoptosis (Beedie et al., 2016). Because of the different
34 476 MW of S and G, the final dose per egg increased as the S/G ratio increases. The results showed
35 477 that the highest mortality rate among three mixture treatments was detected in the higher dose of
36 478 the S/G=2 group (480 µg/kg, the biggest S/G in the test) at 25%. Importantly, in the S/G=0.5
37 479 treatments, the lower dose led to a higher death and malformation rate than in the higher dose
38 480 group, which suggested the NMDR for S and G mixture. This NMDR effect was also detected
39 481 for other developmental indexes and MDA value for the S/G=0.5 groups. At 44.6 µg/kg dose, the
40 482 S/G=0.5 mixture showed significantly decreased REEW and liver weight, as well as significantly
41 483 increased MDA value ($p < 0.05$), while the findings not determined for higher (446 µg/kg) dose.
42 484 All the findings indicated that effects from lower dose treatments must be evaluated separately,
43 485 and the extrapolation from the monotonic dose-response curve can produce misleading data.
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49 487 Similar toxicity effects were observed during chicken embryo development after exposure to the
50 488 three lignin monomers (with S/G ratios from 0.067 to 0.85). Interestingly, a large variation of the S/G
51 489 ratio existed between two of the test samples, with S/G=0.067 for pine while S/G=0.76 for
52 490 Miscanthus. Both pine and Miscanthus showed a high mortality rate at one test dose at 25%. In
53 491 addition, the significantly increased lipid peroxidation level was detected in the higher dose of
54 492 pine monomer group (467 µg/kg), which had the lowest S/G ratio value (0.067). For the three
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3 493 lignin monomers, poplar (146 g/mol) had a slightly higher MW than pine and MC (both at 140
4 494 g/mol), and they had similar mortality and deformation rate in the chicken embryonic assay.
5 495 Clearly, no apparent associations are between S/G ratios (or the mixture concentration) and the
6 496 chicken developmental toxicities for the three plant monomers, and more research are still needed
7 497 to understand the impacts of S/G ratios on the developmental toxicity.
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10 499 Although the relationship between lignin quantities (monolignols) and application in pulping
11 500 processing efficiency, biofuel, and forage digestibility has been widely understood, the
12 501 toxicology effects of the different structure of monolignols are rarely studied (Ayyachamy,
13 502 Cliffe, Coyne, Collier, & Tuohy, 2013). The toxicity study for these monolignols are important
14 503 especially after biosourced polyphenols have been regarded as promising alternatives to
15 504 petroleum-based phenol to produce various bio-based thermosets (Fulcrand, Rouméas, Billerach,
16 505 Aouf, & Dubreucq, 2019). Further studies on the effects of different catalysis reactions on
17 506 polymer toxicity need to be conducted. Moreover, the data of human exposure levels on
18 507 monolignols and lignin monomers are still rare, which are important for their toxicology
19 508 evaluation.
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21 510 **5. Conclusion**

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23 511 Overall, our study revealed that the syringol and guaiacol had different toxicity responses and
24 512 safety warnings on mutagenicity and developmental toxicity of chicken embryos. Syringol had
25 513 signs of mutagenicity in TA98 and TA102 strains, while guaiacol did not. In addition, both
26 514 showed developmental toxicity by the chicken embryonic assay, and guaiacol had a higher
27 515 adverse effect than syringol including alternation of developmental indexes and increased MDA
28 516 value. Moreover, a similar trend existed in the evaluation of three S/G mixtures at different ratios
29 517 (0.5-2). Compared with the *in silico* results, our study demonstrated that the T.E.S.T. simulation
30 518 provided useful screening information for further toxicology studies. Regarding the three lignin
31 519 monomers from biomass, the developmental toxicities were detected, and pine (S/G=0.067)
32 520 revealed the highest adverse effect, including a higher mortality rate and increased lipid
33 521 peroxidation level (at 467 µg/kg). Moreover, different MI values were detected for the three
34 522 lignin monomers, among which the poplar (S/G=0.85) had the highest MI up to 1.8, suggesting
35 523 the sign of mutagenic activity. To the best of our knowledge, this is the first attempt to connect
36 524 the S/G ratio in lignin monomers with the toxicology study and demonstrated the safety concern
37 525 of syringol, guaiacyl, S/G mixtures, and lignin monomers (poplar, pine and miscanthus). Future
38 526 studies should be conducted to reveal the underlying mechanisms involved in chicken embryonic
39 527 developmental toxicity and the impacts of substitution groups on toxicities of S and G.
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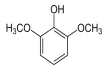

686 Disclaimer

27 687 The authors declare that they have no known competing financial interests or personal
28 688 relationships that could have appeared to influence the work reported in this paper.
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690 Author Contribution Statement

33 691 Xinwen Zhang: Conceptualization, Methodology, Formal analysis, Investigation, Data curation,
34 692 Writing - original draft, Writing - review & editing. Delphis F. Levia: Conceptualization,
35 693 Writing - review & editing. Elvis Osamudiamhen: Methodology, Investigation, and Writing -
36 694 review & editing. Dionisios G. Vlachos: Conceptualization, Methodology, and Writing - review
37 695 & editing. Jeffrey Chang: Conceptualization and Methodology. Changqing Wu:
38 696 Conceptualization, Methodology, Data curation, Supervision, Writing - review & editing, Project
39 697 administration.
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699 Table 1 Acute toxicity, developmental toxicity, and mutagenicity of syringol and guaiacol
700 simulated by Toxicity Estimation Software Tool (T.E.S.T).

Chemical	Structure	Oral rat LD50		Bioaccumulation factor		Developmental Toxicity		Mutagenicity		Acute toxicity chemical classification
		Oral rat LD50 - Log10(mol/kg)	Oral rat LD50 mg/kg	Bioaccumulation factor Log10	Bioaccumulation factor	DT value	DT result	MT value	MT result	
Syringol		2.31	755.83	0.89	7.76	0.54	Developmental toxicant	0.55	Positive	Class 4
Guaiacol		2.42	468.73	0.87	7.44	0.71	Developmental toxicant	0.11	Negative	Class 4

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703 Table 2 Results of the Ames test conducted with guaiacol, syringol, and S/G mixture (0.01 to 1
704 mM at 0.05 mL to yield final dose from 50 to 0.5 nmol/plate). 0.1% DMSO in PBS was used as
705 negative control and used for dissolving test chemicals.

Treatments	Number of revertants/ plate in <i>S. typhimurium</i> strains (M ± 1SD) and (MI)					
	TA 98 (-/+)		TA 100 (-/+)		TA 102 (-/+)	
Negative control (0.1% DMSO)	11 ± 2	31 ± 2	102 ± 4	97 ± 3	223 ± 13	307 ± 9
Positive control	365 ± 32 ^{a**}	456 ± 16 ^{d**}	517 ± 21 ^{b**}	708 ± 6 ^{d**}	749 ± 21 ^{c**}	743 ± 31 ^{d**}
Guaiacol (nmol/plate)						
50	7 ± 4 (0.6)	32 ± 2 (1.0)	130 ± 4 (1.3)	111 ± 2 (1.1)	233 ± 14 (1.0)	315 ± 4 (1.0)
5	8 ± 4 (0.7)	30 ± 3 (1.0)	114 ± 5 (1.1)	120 ± 2 (1.2)	206 ± 6 (0.9)	302 ± 8 (1.0)
0.5	7 ± 1 (0.6)	34 ± 4 (1.1)	102 ± 10 (1.0)	114 ± 4 (1.2)	188 ± 14 (0.8)	307 ± 4 (1.0)
Syringol (nmol/plate)						
50	13 ± 3 (1.2)	41 ± 1* (1.3)	112 ± 3 (1.1)	111 ± 4 (1.1)	288 ± 12* (1.3)	310 ± 3 (1.0)
5	12 ± 3 (1.1)	37 ± 1 (1.2)	103 ± 8 (1.0)	90 ± 8 (0.9)	251 ± 16 (1.1)	387 ± 13* (1.3)
0.5	13 ± 1 (1.2)	38 ± 2 (1.2)	117 ± 6 (1.1)	106 ± 6 (1.1)	269 ± 3 (1.2)	383 ± 6 (1.2)
S/G=0.5 (nmol/plate)						
50	10 ± 3 (0.9)	28 ± 1 (0.9)	85 ± 9 (0.8)	107 ± 3 (1.1)	189 ± 18 [#] (0.8)	320 ± 5 (1.0)
5	8 ± 1 (0.7)	35 ± 2 (1.1)	85 ± 4 (0.8)	103 ± 3 (1.1)	210 ± 16 (0.9)	291 ± 10 (0.9)
0.5	9 ± 3 (0.8)	33 ± 3 (1.1)	91 ± 4 (0.9)	103 ± 7 (1.1)	209 ± 13 (0.9)	299 ± 5 (1.0)
S/G=1 (nmol/plate)						
50	15 ± 3 (1.4)	27 ± 2 (0.9)	100 ± 5 (1.0)	100 ± 5 (1.0)	274 ± 3 (1.2)	317 ± 7 (1.0)
5	14 ± 2 (1.2)	21 ± 4 (0.7)	104 ± 6 (1.0)	111 ± 2 (1.1)	293 ± 8* (1.3)	311 ± 4 (1.0)
0.5	15 ± 1 (1.3)	28 ± 6 (0.9)	102 ± 7 (1.0)	106 ± 5 (1.1)	277 ± 14 (1.2)	327 ± 8 (1.1)
S/G=2 (nmol/plate)						
50	15 ± 4 (1.3)	28 ± 2 (0.9)	110 ± 3 (1.1)	108 ± 5 (1.1)	291 ± 14* (1.3)	318 ± 23 (1.0)
5	13 ± 3 (1.2)	30 ± 1 (1.0)	92 ± 6 (0.9)	111 ± 6 (1.1)	288 ± 1* (1.3)	316 ± 6 (1.0)
0.5	11 ± 4 (1.0)	35 ± 2 (1.1)	104 ± 3 (1.0)	108 ± 14 (1.1)	273 ± 8 (1.2)	337 ± 9 (1.1)

706 Differences were evaluated using one-way ANOVA followed by the Tukey's test and statistical
707 significance was indicated by * $p < 0.05$ and ** $p < 0.01$ when compared to the negative control. #
708 indicated the Significant difference between S/G=0.5 and S/G=2 groups. Data was shown as mean ±
709 standard deviation (M ± 1 SD) revertants/ plate from two independent trials. Positive controls: ^a 2-NF
710 (1 µg/plate), ^b NaN3 (1 µg/plate), ^c Mitomycin C (1 µg/plate), and ^d 2-AA (5 µg/plate).

712 Table 3 Results of the Ames test conducted with MC, Pine, and Poplar (0.01 to 1 mM at 0.05 mL
 713 to yield final dose from 50 to 0.5 nmol/plate) from two independent experiments. 0.1% DMSO in
 714 PBS was used as negative control and used for dissolving test chemicals.

Treatments	Number of revertants/ plate in <i>S. typhimurium</i> strains (M ± 1 SD) and (MI)					
	TA 98 (-/+)		TA 100 (-/+)		TA 102 (-/+)	
Negative control (0.1% DMSO)	24 ± 1	21 ± 1	55 ± 2	88 ± 13	312 ± 14	239 ± 37
Positive control	299 ± 17 ^{a**}	322 ± 29 ^{d**}	631 ± 27 ^{b**}	684 ± 15 ^{d**}	743 ± 28 ^{c**}	716 ± 25 ^{d**}
MC (S/G=0.76) (nmol/plate)						
50	31 ± 3 (1.3)	22 ± 8 (1.0)	68 ± 10 (1.2)	92 ± 13 (1.0)	331 ± 4 (1.1)	227 ± 6 (0.9)
5	32 ± 3 (1.3)	20 ± 7 (1.0)	49 ± 5 (0.9)	87 ± 8 (1.0)	332 ± 16 (1.1)	228 ± 25 (1.0)
0.5	31 ± 1 (1.3)	20 ± 7 (1.0)	53 ± 4 (1.0)	83 ± 6 (0.9)	321 ± 27 (1.0)	221 ± 18 (0.9)
Pine (S/G=0.067) (nmol/plate)						
50	21 ± 2 [#] (0.9)	10 ± 1 [#] (0.5)	58 ± 8 (1.0)	93 ± 7 (1.1)	292 ± 6 [#] (0.9)	183 ± 9 (0.8)
5	26 ± 1 (1.1)	16 ± 3 (0.8)	50 ± 4 (0.9)	89 ± 4 (1.0)	291 ± 15 (0.9)	194 ± 6 (0.8)
0.5	27 ± 1 (1.1)	23 ± 3 (1.1)	49 ± 4 (0.9)	81 ± 4 (0.9)	311 ± 4 (1.0)	193 ± 7 (0.8)
Poplar (S/G=0.85) (nmol/plate)						
50	44 ± 6 [#] (1.8)	24 ± 6 [#] (1.1)	64 ± 3 (1.2)	100 ± 4 (1.1)	371 ± 15 [#] (1.2)	263 ± 33 (1.1)
5	40 ± 6 (1.7)	23 ± 2 (1.1)	49 ± 7 (0.9)	92 ± 2 (1.0)	355 ± 10 (1.1)	243 ± 28 (1.0)
0.5	38 ± 8 (1.6)	28 ± 1 (1.4)	57 ± 1 (1.0)	90 ± 3 (1.0)	326 ± 20 (1.0)	256 ± 35 (1.1)

715 Differences were evaluated using one-way ANOVA followed by the Tukey's test and statistical
 716 significance was indicated by * $p < 0.05$ and ** $p < 0.01$ when compared to the negative control. #
 717 indicated the significantly difference between pine and poplar treatments. Data shown as mean ± standard
 718 deviation revertants/ plate for two replicates for each concentration in each experiment. Positive controls:
 719 ^a 2-NF (1 µg/plate), ^b NaN₃ (1 µg/plat), ^c Mitomycin C (1 µg/plate), and ^d 2-AA (5 µg/plate).

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721 Table 4 Mortality rate and malformation rate of chicken embryos treated with guaiacol, syringol,
 722 three S/G mixtures (S/G=0.5, S/G=1, S/G=2), and three lignin monomers (pine, MC, and
 723 poplar). The chemical solutions were at 0.1 mM and 1 mM and injected at 0.2 mL into the egg
 724 (average weight 60 g) yielding a final dose in egg below: 513 μg syringol/kg, 51.3 μg
 725 syringol/kg, 413 μg guaiacol/kg, 41.3 μg guaiacol/kg, 467 μg pine/kg, 46.7 μg pine/kg, 467 μg
 726 MC/kg, 46.7 μg MC/kg, 487 μg poplar/kg, and 48.7 μg poplar/kg. The data was summarized
 727 from the data in two trials. The number in parentheses represents the number of dead chicken
 728 embryo or malformation chicken embryo.

Treatment	Injection dose (mM)	Treatment dose in egg ($\mu\text{g}/\text{kg}$)	Σ Fertilized eggs	Mortality rate	Malformation rate
Solvent control	1% DMSO		20	0.0% (0)	0.0% (0)
Guaiacol	0.1	41.3	8	12.5% (1)	0.0% (0)
	1	413	8	12.5% (1)	25% (2)
Syringol	0.1	51.3	8	12.5% (1)	0.0% (0)
	1	513	8	0.0% (0)	0.0% (0)
S/G=0.5	0.1	44.6	8	12.5% (1)	12.5% (1)
	1	446	12	8.3% (1)	0.0% (0)
S/G=1	0.1	46.3	8	0.0% (0)	0.0% (0)
	1	463	12	8.3% (1)	0.0% (0)
S/G=2	0.1	48	8	12.5% (1)	0.0% (0)
	1	480	12	25.0% (3)	8.3% (1)
Pine (S/G=0.067)	0.1	46.7	8	12.5% (1)	0.0% (0)
	1	467	8	25.0% (2)	0.0% (0)
MC (S/G=0.76)	0.1	46.7	8	25.0% (2)	0.0% (0)
	1	467	8	0.0% (0)	0.0% (0)
Poplar (S/G=0.85)	0.1	48.7	8	12.5% (1)	0.0% (0)
	1	487	8	12.5% (1)	0.0% (0)

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731 Table 5 The ratio of the embryo to egg weight (REEW), liver somatic index (LSI, %), and
 732 weight of embryo and organs of chicken embryos at day 18 after treatments of guaiacol,
 733 syringol, three mixture (S/G=0.5, S/G=1, S/G=2) and three lignin monomers (pine, MC, and
 734 poplar). The chemical solutions were at 0.1 mM and 1 mM and injected at 0.2 mL into the egg
 735 (average weight 60 g) yielding a final dose in egg below: 513 μg syringol/kg, 51.3 μg
 736 syringol/kg, 413 μg guaiacol/kg, 41.3 μg guaiacol/kg, 467 μg pine/kg, 46.7 μg pine/kg, 467 μg
 737 MC/kg, 46.7 μg MC/kg, 487 μg poplar/kg, and 48.7 μg poplar/kg.

Treatment	Injection dose (mM)	Treatment dose in egg ($\mu\text{g}/\text{kg}$)	REEW	LSI (%)	Weight (g)	
					Liver	heart
Solvent control	1% DMSO		0.39 ± 0.04	2.41 ± 0.19	0.56 ± 0.05	0.23 ± 0.03
Guaiacol	0.1	41.3	0.39 ± 0.02	2.27 ± 0.10	0.53 ± 0.03	0.22 ± 0.01
	1	413	$0.32 \pm 0.04^*$	$1.94 \pm 0.22^*$	$0.37 \pm 0.01^*$	0.17 ± 0.00
Syringol	0.1	51.3	0.40 ± 0.04	2.34 ± 0.20	0.56 ± 0.04	0.21 ± 0.00
	1	513	0.34 ± 0.02	2.48 ± 0.05	0.54 ± 0.07	0.22 ± 0.03
S/G=0.5	0.1	44.6	$0.32 \pm 0.01^*$	2.28 ± 0.29	$0.47 \pm 0.05^*$	0.20 ± 0.03
	1	446	0.36 ± 0.04	2.29 ± 0.01	0.51 ± 0.07	0.19 ± 0.04
S/G=1	0.1	46.3	0.37 ± 0.01	2.24 ± 0.18	0.50 ± 0.00	0.19 ± 0.01
	1	463	0.37 ± 0.01	2.30 ± 0.15	0.55 ± 0.01	0.20 ± 0.01
S/G=2	0.1	48	0.35 ± 0.02	2.43 ± 0.21	0.53 ± 0.02	0.20 ± 0.01
	1	480	0.37 ± 0.06	2.22 ± 0.17	0.52 ± 0.08	0.22 ± 0.02
Pine (S/G=0.067)	0.1	46.7	0.41 ± 0.01	2.10 ± 0.22	0.51 ± 0.05	0.22 ± 0.01
	1	467	0.42 ± 0.01	2.23 ± 0.17	0.53 ± 0.07	0.22 ± 0.01
MC (S/G=0.76)	0.1	46.7	0.44 ± 0.03	2.17 ± 0.22	0.53 ± 0.08	0.22 ± 0.02
	1	467	0.41 ± 0.02	2.07 ± 0.16	0.50 ± 0.07	0.22 ± 0.01
Poplar (S/G=0.85)	0.1	48.7	0.41 ± 0.02	2.15 ± 0.09	0.51 ± 0.03	0.22 ± 0.00
	1	487	0.43 ± 0.03	2.20 ± 0.14	0.52 ± 0.02	0.21 ± 0.01

738 Differences were evaluated by one-way ANOVA and followed by the Tukey's test, and statistical
 739 significance was indicated by $p < 0.05$ (* means significant difference compared to the solvent control; #
 740 means significant difference between different S/G ratio at the same dose). REEW: ratio of embryo to egg
 741 weight, LSI: liver somatic index. All values are expressed as mean \pm 1 SD from two independent trials.

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4 **The impact of differential lignin S/G ratios on mutagenicity and chicken**
5 **embryonic toxicity**

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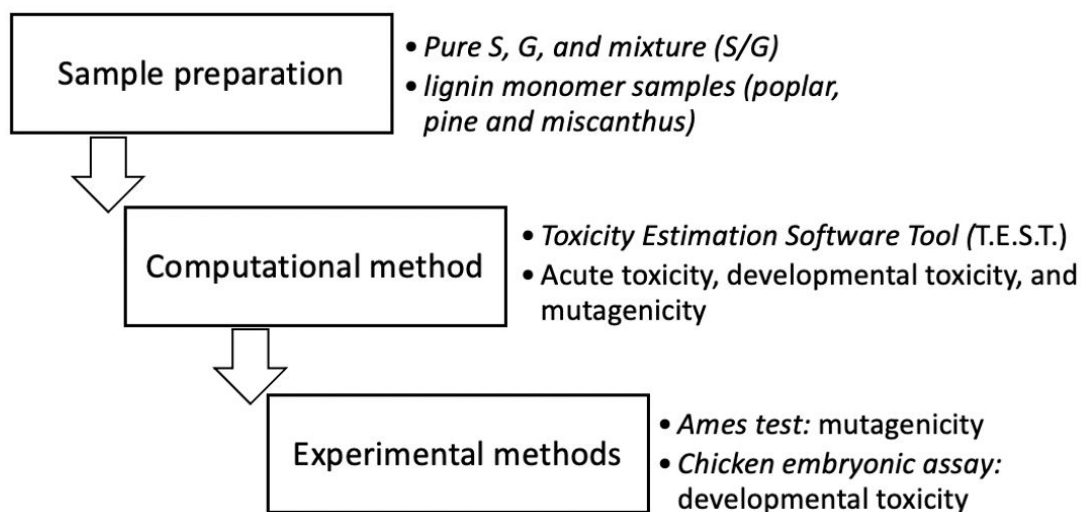


Figure 1 Scheme showing multitiered toxicology evaluation of S/G mixture and lignin monomers

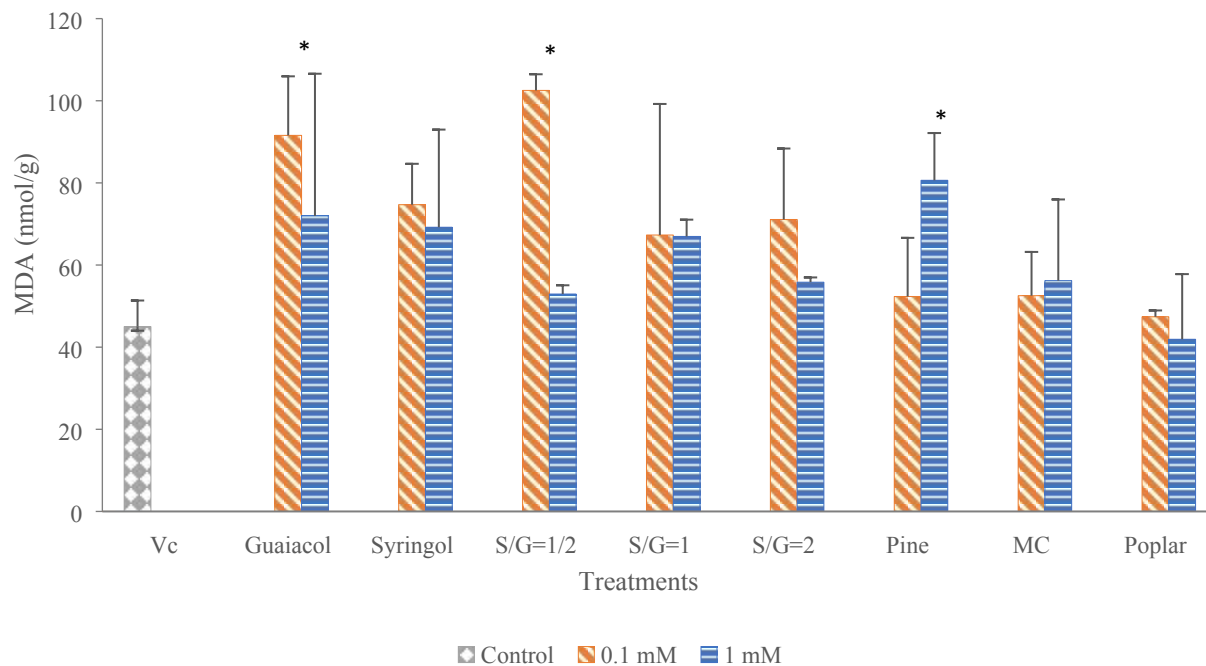


Figure 2 Impacts of Guaiacol, Syringol, three ratios of S/G mixture (S/G=0.2, S/G=1, S/G=2) and three lignin monomers (pine, MC, and poplar) on malondialdehyde (MDA) of livers in chicken embryos. The chemical solutions were at 0.1 mM and 1 mM and injected at 0.2 mL into the egg (average weight 60 g) yielding a final dose in egg below: 513 μg syringol/kg, 51.3 μg syringol/kg, 413 μg guaiacol/kg, 41.3 μg guaiacol/kg, 467 μg pine/kg, 46.7 μg pine/kg, 467 μg MC/kg, 46.7 μg MC/kg, 487 μg poplar/kg, and 48.7 μg poplar/kg. Values are expressed as mean \pm 1 SD from two independent trials. Differences were evaluated using one-way ANOVA followed by the Tukey's test between two doses treatment groups and solvent control, and statistical significance was indicated by $p < 0.05$ (* $p < 0.05$). # means there was a significant difference between two dosages in same treatment

Graphical Abstract

