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

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Abstract

Lignin and lignin-based materials have received considerable attention in various fields due to their promise as sustainable feedstocks. Guaiacol (G) and syringol (S) are two primary monolignols that occur in different ratios for different plant species. As methoxyphenols, G and S have been targeted as atmospheric pollutants and their acute toxicity examined. However, there is a rare understanding of the toxicological properties on other endpoints and mixture effects of these monolignols. To fill this knowledge gap, our study investigated the impact of different S/G ratios (0.5, 1, and 2) and three lignin depolymerization samples from poplar, pine, and miscanthus species on mutagenicity and developmental toxicity. A multi-tiered method consisted with *in silico* simulation, *in vitro* Ames test, and *in vivo* chicken embryonic assay was employed. In the Ames test, syringol showed a sign of mutagenicity, while guaiacol did not, which agreed with the T.E.S.T. simulation. For three S and G mixture and lignin monomers, mutagenic activity was related to the proportion of syringol. In addition, both S and G showed developmental toxicity in the chicken embryonic assay and T.E.S.T. simulation, and guaiacol had a severe effect on lipid peroxidation. A similar trend and comparable developmental toxicity levels were detected for S and G mixtures and the three lignin depolymerized monomers. This study provides data and insights on the differential toxicity of varying S/G ratios for some important building blocks for bio-based materials.

Short abstract

To understand the toxicity impacts of S/G ratios from various lignocellulosic biomass feedstocks, we used a multi-tiered platform to study the toxicities of the pure S and G, mixtures at different S/G ratios, and three lignin depolymerization samples. In the Ames test, syringol showed a sign of mutagenicity while guaiacol did not, which agreed with the T.E.S.T. The S/G mixtures and lignin samples revealed the importance of the S/G ratio on mutagenicity in the Ames test, and developmental toxicity in the chicken embryos.

Key words

Guaiacol; syringol; lignin; developmental toxicity; mutagenicity; in silico simulation

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,

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

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

2. Methods

2.1 Chemicals and materials

All biomass samples were obtained from the Idaho National Laboratory and fully characterized using the NREL LAP protocols. The samples were milled to particles ranging from 0.42 mm (40 mesh) – 2 (10 mesh) mm by Forest Concepts and used as received. 5 wt.% Ru/C powder was purchased from Sigma Aldrich and used as received. Methanol (certified ACS Reagent Grade, 99.8%) was purchased from Fisher Chemicals and used as received. DMSO-d_6, pyridine-d_5 were purchased from Sigma Aldrich. Deionized water (Millipore model Direct Q3 UV R) was used for

all preparations requiring water. 17β-estradiol (E2), dimethyl sulfoxide (DMSO) (D1391), and phosphate buffered solution (PBS) (Gibco, 20-012-027) were purchased from Fisher Scientific (Waltham, MA, USA). The three Salmonella typhimurium tester strains (TA 98, TA 100, and TA 102), top agar, Oxoid Nutrient Broth No.2, S9 mixture solutions were purchased from Molecular Toxicology Inc (Boone, NC, USA).

The molecular weight (MW) for syringol and guaiacol were 154.16 and 124.14 g/mol. The mixtures of S and G at three different ratios (0.5, 1, and 2) were prepared by mixing the S and G ratio mole at 1 M and then diluted to the final dose. The average MW for three S/G mixtures was 134.15, 139.15, and 144.15 g/mol at S/G=0.5, 1, and 2. The average MW for three plant lignin monomers are as below based on the genus: pine (*Pinus* spp.): 140 g/mol, miscanthus (MC, *Miscanthus* spp): 140 g/mol, poplar (*Populus* spp.): 146 g/mol.

2.2 Preparation and characterization of lignin monomer samples

Reductive catalytic fractionation (RCF) of plant lignin. Sample of 40 g of biomass (poplar, pine and miscanthus) was added to 800 ml methanol in a 1.2 L high-pressure Parr reactor along with 4 g Ru/C. The reactor was stirred with a mechanical stirrer and heated with a high-temperature heating jacket connected to a variable power supply controlled by a PID temperature controller and a K-type thermocouple to measure the reaction temperature through a thermowell. Once sealed, the reactor was purged three times with N_2 and then pressurized with 40 bars of H_2. The reactor was heated to 250 °C (it takes ~10 – 15 min to reach the set point) and held for 15 h while stirring. Reaction conditions were optimized in our previous studies (Ebikade et al., 2020; Li et al., 2018; Shuai et al., 2018; S. Wang, Shuai, Saha, Vlachos, & Epps, 2018). Subsequently, the reactor was cooled until reaching room temperature and the gas phase was released. A portion of the reaction products was filtered for monomer identification and quantification. The remaining liquid was filtrated through a nylon membrane filter (Whatman®, 0.2 μm) and the filtrate was stored for further analyses.

Isolation of lignin monomers. After evaporating methanol from the lignin product solution,10 ml of cyclohexane (to remove monomers from the lignin oil) was added to the viscous lignin oil. The mixture was vortexed for 30 seconds and placed in a sonicating bath for 1 hour. The cyclohexane layer was collected for monomer recovery and fresh cyclohexane was added, vortexed and sonicated for two more monomer removal steps. After the three-time cyclohexane extraction, the monomers were recovered following evaporation of cyclohexane.

Nuclear magnetic resonance. Heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectra of extracted lignin oils and isolated oligomer oils were recorded at 25 °C on an Avance III 400 MHz NMR spectrometer (Bruker). Approximately 30 mg of filtered lignin oil was dissolved in 500 μl of premixed DMSO-d_6/pyridine-d_5 (4:1) prepared in quartz NMR tubes (NewEra). Data processing was performed using the Mestrelab Research software (mNOVA).

2.3 Toxicity Estimation Software Tool (T.E.S.T.)

The Toxicity Estimation Software Tool (T.E.S.T.) was developed by United States Environmental Protection Agency (EPA) and used a variety of QSAR methodologies, including Hierarchical, FDA, Single model, Group contribution, Nearest neighbor method, to estimate toxicity and physical properties of test chemicals (Toxicity Estimation Software Tool (TEST) 2016). The predicted toxicity data presented in this study was generated from the Consensus method which was the average of the predicted toxicities of previous five QSAR methods. The endpoints included in our study: Oral rat LD 50, Developmental Toxicity, and Mutagenicity.

2.4 Ames test

The Ames test was conducted by three *Salmonella typhimurium* tester strains (TA 98, TA 100, and TA 102) using a preincubated method as described by Maron & Ames (1983). The TA98 and TA100 strains were suggested as viable alternatives to the current OECD Test Guideline TG471 by several recent studies (Williams et al., 2019; Gao et al., 2021; Khan et al., 2021). We also included the TA102 as an additional test strain in our modified approach. The strains were grown overnight in Oxoid Nutrient Broth No.2 and incubated in a shaking incubator at 37°C and 100 rpm to reach cell densities at 1–2 × 10^9 cells/mL. Each strain was conducted both in the absence and in the presence of a metabolic activation mixture S9. S9 was freshly prepared before each test by addition of liver extracts of Sprague–Dawley rats induced with Aroclor 1254, regensys "A" and "B". The PBS was used as an S9 alternative for the test without S9 activation. Each test compound was firstly dissolved in DMSO and then diluted by PBS buffer to reach the concentration at 0.01 to 1 mM. The 0.05 ml of tested compounds were added to a 0.5 mL of S9 mixture (or 0.5 mL PBS in without S9 mixture). Then 0.1 mL of three bacterial culture added to the mixture and incubated at 37 °C. After 30 min incubation, the 2 mL of top agar was added to each tube and mixed well. The mixture was poured on to a minimal agar plate. The three treatment dosages from 1 to 0.01 mM at 0.05 mL addition in each plate yielded the final concentration from 50 to 0.5 nmol/plate. After 48 h incubation, the His+ revertant colonies on plates were counted manually. Each test was repeated in two independent trials and duplicate for each trial.

2.5 Chicken embryonic assay

Egg treatments. A total of 160 fertilized Leghorn eggs were obtained from the University of Delaware research farm and used in the assay. The chicken embryo as an *in vivo* model using early life stages have been widely used in toxicity assessment (Uggini, Patel, & Balakrishnan, 2012; Mentor, Bornehag, Jönsson, & Mattsson, 2020) The eggs were weighed and divided into 17 groups: vehicle control, and two dosages of guaiacol, syringol, three S/G mixtures (at ratio 0.5, 1, and 2), and three plant lignin monomers (MC, pine, and poplar). The chicken embryos on day 6 were injected with each chemical solution (1 mM and 0.1 mM) or 1% DMSO in PBS (vehicle control) at 0.2 ml using a syringe (1 ml). The final doses in egg (average of 60 g per egg) included: 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. After the treatments, eggs were put back to the egg incubator at 38 ℃ and 60% relative humidity.

***Developmental toxicity* evaluation**. After chemical treatments, the eggs were candled every two days and recorded for dead embryo numbers. The embryos were euthanized on day 18 by placing them in the refrigerator at 4°C overnight. After eggs were opened, all embryos were weighted and recorded for abnormality. Additional measurements were conducted on liver mass and heart mass after embryos were dissected. The liver somatic index (LSI) was calculated as LSI = liver mass/embryo mass × 100%, which reflected the health indicator after embryo exposure to the environmental contaminant. The significantly changed values of LSI ($p < 0.05$) were identified after comparison to the solvent control group, which indicates health problems in the chicken embryo development (Guo et al. 2018; Mentor, Wänn, et al. 2020).

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

2.6 Data analysis

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

3. Results

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

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

syringol. For the mutagenicity, syringol had a higher value at 0.55 than guaiacol (0.11) and was classified as mutagenicity positive. On the other hand, guaiacol was regarded as mutagenicity negative. Interestingly, with an additional CH_3O group in syringol, it has higher mutagenicity but lowers developmental toxicity.

3.2 Different mutagenicity was related with three S/G ratios and three different lignin monomers

The mutagenic activity of guaiacol, syringol, three S/G mixtures (at ratio 0.5, 1, and 2), and three lignin monomers (MC, pine, and poplar) was evaluated by the Ames test at three concentrations (0.5, 5, and 50 nmol/plate). As shown in Table 2 and 3, the positive control of each bacterial strain (TA 98, TA 100, and TA 102) with or without S9, produced statistically significant increases in the number of revertant colonies, and negative controls of three strains were in our historical ranges of number of revertant colonies, which confirmed the sensitivity and accuracy of the test system. Syringol significantly increased revertant number of TA 98 and TA102 strains at 5 and 50 nmol/plate, with MI at 1.3 ($p < 0.05$). In contrast, there was no significant increased number of revertant colonies for guaiacol treatments. The experimental findings agreed quite well with the results from the *in silico* method, T.E.S.T. simulation, as summarized above (Table 1). Among the three S/G mixtures, the S/G ratio at 1 and 2 significantly increased revertant numbers of TA 102 strain without S9 activation ($p < 0.05$), while the mixture with S/G ratio at 0.5 had no significant increase. Additionally, the mixture with a higher S/G ratio (S/G=2) at 50 nmol/plate had a higher number of revertant colonies than the mixture with the lowest S/G at 0.5 at the same concentration for TA 102 strain (at 50 nmol/plate, $p < 0.05$). With MI <2, the increase in the sign of mutagenicity of three mixtures was largely associated with the bigger content of syringol in the mixtures.

As shown in Table 3, the mutagenic activity of three lignin monomers varied in the Ames test, but the higher sign of mutagenicity was still recorded with larger S/G ratios. The poplar lignin monomers, with the highest S/G ratio among these three samples, showed the highest MI values, up to 1.8 at TA 98 strain. After TA 98 strain without S9 activation was treated with the poplar lignin monomer at 0.5 to 50 nmol/plate, the MI levels were between 1.6 to 1.8. When MIs of the TA 98 strain (with and without S9 activation) were compared between the treatments of pine and poplar lignin monomers at 50 nmol/plate, a significantly smaller value of MI was detected in the pine lignin monomer treatment ($p < 0.05$). Similar findings were also determined for the TA 102 revertants when treated with the pine samples at 50 nmol/plate ($p < 0.05$).

3.3 Different S/G ratios impacted differently the chicken embryonic and developmental toxicity

Table 4 summarizes the mortality and malformation number of chicken embryos after exposure to S, G, three S/G mixtures, and three lignin monomers at two injection concentrations (0.1 and 1 mM), which yielded different final dose in eggs due the difference in molecular weight. The fertilized eggs were randomly assigned to each treatment on day 6. Four more fertilized egg were recorded in three mixture groups at high dose injection (1 mM) because one more trial (4 eggs) was included to confirm the findings. One out of eight embryos was dead after each guaiacol treatment, and 25% stunting embryos were detected in the 413 µg/kg guaiacol group, while only one death was found for both syringol groups. Higher developmental toxicity was determined for guaiacol which is in good agreement with the T.E.S.T simulation for both chemicals. The

T.E.S.T. results showed that the syringol and guaiacol were both developmental toxicants, and guaiacol showed a higher toxicity value than the findings of syringol.

There were no clear associations between S/G ratios (or the mixture concentration) and the chicken developmental toxicities. The 12.5% death rates were both recorded for the 0.5 and 2 S/G ratio mixtures groups at the lower dosage, while 8.3% and 25% were determined for the higher dose respectively. Interestingly, the lowest death rates were determined for the middle ratio mixture (S/G=1) at 0 and 8.3% for 46.3 and 463 μg/kg, respectively. After exposure to the three lignin monomer samples, different chicken mortality was determined for each plant lignin monomer sample, with the highest death at 25% after exposure to the higher dose of pine (467 μg/kg) and the lower dose of miscanthus (46.7 μg/kg) lignin monomers. The pine lignin with the smallest S/G ratio at 0.067 indicated the highest proportion of guaiacol. Guaiacol had higher developmental toxicity than the findings in syringol, as shown in Tables 1 and 4, which might explain the higher developmental toxicity in the pine samples compared with the findings from the other two plant samples.

As shown in Table 5, the ratio of embryo weight to egg weight (REEW) decreased after exposure to higher dose of guaiacol (413 μg/kg) and lower dose of S/G=0.5 mixture (44.6 μg/kg) ($p < 0.05$). The LSI was calculated for each group and served as a general indicator of health response from exposure to an environmental contaminant. The higher dose of guaiacol (413 μg/kg) decreased the LSI values significantly more than the solvent control ($p < 0.05$). Smaller REEW also resulted in significantly lower LSI values. The three lignin monomer mixtures had similar REEW and LSI. Additionally, the liver weight for each treatment also reflected the toxic impacts from the same chemical treatments. Exposure to guaiacol (413 μg/kg) and the mixture at S/G=0.5 (44.6 μg/kg) resulted in significantly decreased liver weights ($p < 0.05$) compared to the value in the solvent control group, and there was no difference among the chicken heart weights from all the treatments. The chicken liver was the first organ to respond to containments and might be impacted more than chicken hearts.

The MDA levels for each treatment group were determined to evaluate the oxidative stress level of fetal chicken livers (Fig. 2). The solvent control (1% DMSO) showed the lowest MDA level at 45.01 ± 6.35. Guaiacol and the S/G=0.5 mixture at the lower concentration significantly increased the MDA level than the MDA value in the control group, at 91.57 ± 14.38 and 102.52 ± 3.93, respectively ($p < 0.05$). The higher MDA values indicated more oxidative stress, contributing to lower REEW and LSI in the lower dose of S/G mixture (S/G=0.5). Moreover, among the three lignin monomers, a significantly increased MDA value was detected in the pine group, which has the lowest S/G ratio at 0.067 ($p < 0.05$). With guaiacol having higher developmental toxicity and MDA values than the findings in syringol (Table 1, 4, and 5, Fig. 2), pine samples showed the highest MDA when compared with the findings from the other two plant samples with larger S/G ratios. The greater oxidative stress in the treatment of the pine samples could be associated with the highest chicken embryonic death (Table 4) among all 3 test plant lignin monomers.

4. Discussion

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

Besides serving as precursors of plastic polymer, the methoxyphenols (MPs) were regarded as atmosphere pollutants generated from lignin pyrolysis during biomass burning (Collard & Blin, 2014). The 2-methoxyphenol (guaiacol), 2, 6-dimethoxyphenol (syringol), along with their derivatives, were prominent types of MPs existing in lignin. As biomarkers for woodsmoke exposure, both of them have been detected in human urine at concentrations of 8 μg/m^3 (Dills et al., 2006). However, there was little knowledge about the toxicity of guaiacol and syringol, except their basic acute toxicity. Guaiacol has been regarded as harmful for aquatic organisms, which belong to the 'harmful' and 'slightly toxic' hazard classes using luminescence test according to the European and American legislation, respectively (Pflieger & Kroflič, 2017). Furthermore, belong to the MPs, syringol had one more methoxy group them guaiacol, which attributed to their potential different toxicology profiles. One previous study revealed that methoxyphenols with a shorter alkyl chain showed weaker aquatic toxicity than longer alkyl chains. In specific, compared with guaiacol, two 4-substituted guaiacols, creosol (4-methylguaiacol) and 4-ethylguaiacol (4-EG) had higher toxicity on green algae, daphnia, and fish (Wei et al., 2018). Therefore, in this study, we investigated two critical toxicity endpoints: mutagenic activity and the developmental toxicity of guaiacol, syringol, and three mixtures. Additionally, we extended the assessment to lignin monomers isolated from three test species (poplar, pine, and miscanthus).

In T.E.S.T. simulation, the guaiacol had higher acute toxicity than syringol, and both of them were classified in Acute toxicity class 4. The oral rat LD50 value of guaiacol was 468.73 mg/kg and 755.83 mg/kg for syringol (Table 1). Compared with the existing experimental oral rat LD50 for guaiacol at 520 mg/kg and syringol at 550 mg/kg (Orłowski & Boruszak, 1991), the oral rat LD50 of guaiacol from T.E.S.T had even lower value than reported experimental data while syringol had higher values than the experimental values. In addition, in the rat test, guaiacol only produced slightly higher acute toxicity than that from syringol, while the difference of oral rat LD50 between guaiacol and syringol was bigger from the T.E.S.T. simulation. Regarding the mutagenicity, syringol showed a higher mutagenicity value and belonged to the mutagenicity positive, while guaiacol belonged to the mutagenicity negative. The finding of higher potential mutagenicity of syringol than guaiacol from *in vitro* Ames test was consistent with the simulated mutagenicity results. In the Ames test, the revertant numbers of each solvent control were within the historical ranges and control limits of our laboratory and agreed with values reported in the literature (Levy et al., 2019). We applied the mean ± 2 standard deviations as our control limits suggested by Kato et al. (2018). The higher MI values (up to 1.3) were detected after exposure to syringol than guaiacol at TA 98 and TA 102 strains (5 and 50 nmol/plate). The mutagenicity of syringol and guaiacol at higher concentrations (30 μmol/plate) was evaluated in a previous study as tobacco smoke constituents and no mutagenic activity was reported (Florin, Rutberg, Curvall, & Enzell, 1980). Regarding the developmental toxicity, T.E.S.T finding showed that guaiacol had a higher developmental toxicity value (0.71) than syringol (0.54), and they all belonged to the developmental toxicants. To confirm the simulation results, we applied a chicken embryo model for assessment, which serves as a promising alternative method to traditional animal studies (Samak et al., 2020). In this study, the adverse effects of chicken embryogenesis were observed after exposure to both guaiacol and syringol at two doses. The 12.5% death rates were detected for two doses of guaiacol (41.3 and 413 μg/kg) and the lower dose of syringol (51.3 μg/kg) groups. Additionally, 25% of deformed embryos (stunting) were found after exposure to the 413 μg guaiacol/kg. Due to the different MW between guaiacol (124.14 g/mol) and syringol

(154.16 g/mol), they had different final doses in egg even from the same injection concentrations (0.1 and 1 mM). Higher doses per egg in μg/kg were observed in syringol than in guaiacol, but guaiacol still showed higher mortality as well as higher deformation rate at higher dose group. Additionally, the higher mortality rate was observed in the lower dose of syringol group, which indicated the presence of non-monotonic dose response (NMDR) effects (Vandenberg et al., 2012). The NMDR included different shape of dose-response curves than the traditional one, which means that the lower dose might have a larger effect than that of the higher dose.

4.2 Mutagenicity increased with higher S/G ratio among three different S/G ratios and three different lignin monomers

Lignin-based polymer materials have been reported increasingly applied as an alternative green materials (Kai et al., 2016; C. Wang, Kelley, & Venditti, 2016). However, the toxicology data was limited to single hazard components present in the lignin, and the assessment of a set of toxicity properties was absent, which includes different aspects (acute toxicity, mutagenicity, and developmental toxicity). Poplar, pine and miscanthus are promising lignocellulosic feedstocks on the production of biofuels and biomaterials (Sannigrahi, Ragauskas, & Tuskan, 2010). As a phenolic polymer, lignin is composed principally of three alcohols (p-coumaryl, coniferyl, and sinapyl) and generated as different structural units (H, G, and S) by polymerization (A. C. dos Santos, Ximenes, Kim, & Ladisch, 2019). The prooxidant activity and toxic effect also exist in plant phenolic compounds, such as flavonoids and lignin precursors (Sakihama, Cohen, Grace, & Yamasaki, 2002). In this study, we extended the mutagenicity assessment to three S/G mixtures (from 0.5 to 2) and three lignin monomers (pine, miscanthus, and poplar) at different S/G ratios (from 0.067 to 0.85). We specifically focused on relatively low exposure levels (at 0.001 to 1 mM) that may be easily overlooked but are nonetheless important for evaluation since they could be related to human potential exposure levels with potential NMDR behavior and low dose effects. The NMDR behavior of genotoxicity has been reported using *in vivo* comet assay in a zebrafish embryo model for freshwater sediment samples due to overlapping cytotoxic effects (Garcia-Käufer et al., 2015). In addition, the antimicrobial effects of the three lignin depolymerized monomers were detected. The minimal inhibitory concentrations against *S. aureu* and *E. coli* were at 2.5 mg/mL and thus limited the high test concentrations in the Ames tests. In this study, the highest and significantly different MI values indicating signs of mutagenicity were observed at middle doses for syringol and S/G=1 mixture in TA 102 strain, with S9 activation and without S9, respectively. In agreement with pure S and G data, the mixtures and lignin samples with higher S ratios showed a higher mutagenic index. Significantly increased revertant numbers were detected for S/G equal to 2 and 1 treatments ($p < 0.05$), but not in the group with lower S/G value (S/G=0.5). For the three lignin monomers, a higher MI value up to 1.8 was observed after exposure to poplar (S/G=0.85) at 50 nmol/plate for the TA 98 strain, showing a significant sign of mutagenicity. Furthermore, compared with poplar treatments, the pine with the lowest S/G ratio showed significantly reduced revertant numbers for the TA 98 and TA 102 strains. Because TA 98 and TA 1537 detect frame shifts, while TA 100 and TA 1535 detect mutagens which cause base-pair substitutions, and TA 102 detects transition mutagens containing nucleotides AT (Vijay et al. 2018), it is possible to just use the three strains— TA 98, TA 100, and TA 102— in this study for the examination of frame shifts, base-pair substitutions and transition mutagens containing nucleotides AT, respectively. Williams et al (2019) suggested that test strains TA98 and TA100 were enough for detecting most bacterial mutagens (93%); while including an *in vitro* assay that detects clastogens, such as the *in vitro* chromosome

aberration assay, would detect 99% of bacterial mutagens. With the addition of TA 102 in our current approach, we could determine higher bacterial mutagens than that suggested by Williams et al (2019). This study is only the first stage for toxicity evaluation of these biomass-based materials using a multi-tiered platform including *in silico* simulation, *in vitro* Ames test, and *in vivo* chicken embryonic assay. In this study we target most bacterial mutagens. For our future extensive mutagenicity studies, we will use the recommendation from the updated OECD TG 471(OECD, 2020) for the compounds or mixture showing high toxicity risks, and we fully understand the benefits of using the five strains as they can test the wide possibility of mutagenicity. In addition, we will include other in vivo mutagenicity tests, such as Comet assay, to assess the genotoxicity more comprehensively and detect potential NMDR in a wider dosage range.

4.3 Varying S/G ratios differentially impacted the chicken embryonic and developmental toxicity

Furthermore, the developmental toxicity of phenolic compounds has been reported in different models (Yang et al., 2018; Chao et al., 2020). In our results, syringol and guaiacol showed different toxicity profiles on the two endpoints: mutagenicity and developmental toxicity. The adverse effects on chicken embryonic development were observed in both S and G compounds. The three S/G mixtures groups all increased embryo mortality, with the highest death rate observed at the higher dose of S/G=2 group at 25%. Additionally, the significant alteration of several developmental indexes, including small REEW and liver weight, was detected at the lower dose of the S/G=0.5 mixture. Moreover, a significantly increased MDA level, as a biomarker of lipid peroxidation, in the liver sample was observed in the same group compared to the control (Kurantowicz et al., 2017). The one potential mechanism of adverse effects on embryonic development was related to oxidative stress damage(Nguyen et al., 2020). For the S/G=2 treatments, the high mortality and malformation rates did not exist with alternated other developmental indexes or increased MDA level, which suggested that other mechanisms might play roles, such as anti-angiogenic or apoptosis (Beedie et al., 2016). Because of the different MW of S and G, the final dose per egg increased as the S/G ratio increases. The results showed that the highest mortality rate among three mixture treatments was detected in the higher dose of the S/G=2 group (480 μg/kg, the biggest S/G in the test) at 25%. Importantly, in the S/G=0.5 treatments, the lower dose led to a higher death and malformation rate than in the higher dose group, which suggested the NMDR for S and G mixture. This NMDR effect was also detected for other developmental indexes and MDA value for the S/G=0.5 groups. At 44.6 μg/kg dose, the S/G=0.5 mixture showed significantly decreased REEW and liver weight, as well as significantly increased MDA value ($p < 0.05$), while the findings not determined for higher (446 μg/kg) dose. All the findings indicated that effects from lower dose treatments must be evaluated separately, and the extrapolation from the monotonic dose-response curve can produce misleading data.

Similar toxicity effects were observed during chicken embryo development after exposure to the three lignin monomers (with S/G ratios from 0.067 to 0.85). Interestingly, a large variation of the S/G ratio existed between two of the test samples, with S/G=0.067 for pine while S/G=0.76 for Miscanthus. Both pine and Miscanthus showed a high mortality rate at one test dose at 25%. In addition, the significantly increased lipid peroxidation level was detected in the higher dose of pine monomer group (467 μg/kg), which had the lowest S/G ratio value (0.067). For the three

lignin monomers, poplar (146 g/mol) had a slightly higher MW than pine and MC (both at 140 g/mol), and they had similar mortality and deformation rate in the chicken embryonic assay. Clearly, no apparent associations are between S/G ratios (or the mixture concentration) and the chicken developmental toxicities for the three plant monomers, and more research are still needed to understand the impacts of S/G ratios on the developmental toxicity.

Although the relationship between lignin quantities (monolignols) and application in pulping processing efficiency, biofuel, and forage digestibility has been widely understood, the toxicology effects of the different structure of monolignols are rarely studied (Ayyachamy, Cliffe, Coyne, Collier, & Tuohy, 2013). The toxicity study for these monolignols are important especially after biosourced polyphenols have been regarded as promising alternatives to petroleum-based phenol to produce various bio-based thermosets (Fulcrand, Rouméas, Billerach, Aouf, & Dubreucq, 2019). Further studies on the effects of different catalysis reactions on polymer toxicity need to be conducted. Moreover, the data of human exposure levels on monolignols and lignin monomers are still rare, which are important for their toxicology evaluation.

5. Conclusion

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

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Author Contribution Statement

Xinwen Zhang: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing. Delphis F. Levia: Conceptualization, Writing - review & editing. Elvis Osamudiamhen: Methodology, Investigation, and Writing - review & editing. Dionisios G. Vlachos: Conceptualization, Methodology, and Writing - review & editing. Jeffrey Chang: Conceptualization and Methodology. Changqing Wu: Conceptualization, Methodology, Data curation, Supervision, Writing - review & editing, Project administration.

Table 1 Acute toxicity, developmental toxicity, and mutagenicity of syringol and guaiacol simulated by Toxicity Estimation Software Tool (T.E.S.T).

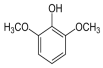
Chemical	Structure	Oral rat LD50		Bioaccumulation factor		Developmental Toxicity		Mutagenicity		Acute toxicity chemical classification
		Oral rat LD50 - Log10(mol/kg)	Oral rat LD50 mg/kg	Bioaccumulation factor Log10	Bioaccumulation factor	DT value	DT result	MT value	MT result	
Syringol		2.31	755.83	0.89	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

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

Treatments	Number of revertants/ plate in S. typhimurium 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)

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

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

Treatments	Number of revertants/ plate in S. typhimurium 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)

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

Table 4 Mortality rate and malformation rate of chicken embryos treated with guaiacol, syringol, three S/G mixtures (S/G=0.5, S/G=1, S/G=2), and three lignin monomers (pine, MC, and poplar). 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. The data was summarized from the data in two trials. The number in parentheses represents the number of dead chicken embryo or malformation chicken embryo.

Treatment	Injection dose (mM)	Treatment dose in egg (μg/kg)	∑ 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)

Table 5 The ratio of the embryo to egg weight (REEW), liver somatic index (LSI, %), and weight of embryo and organs of chicken embryos at day 18 after treatments of guaiacol, syringol, three mixture (S/G=0.5, S/G=1, S/G=2) and three lignin monomers (pine, MC, and poplar). 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.

Treatment	Injection dose (mM)	Treatment dose in egg (μg/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 ± 0.04*	1.94 ± 0.22*	0.37 ± 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 ± 0.01*	2.28 ± 0.29	0.47 ± 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

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

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

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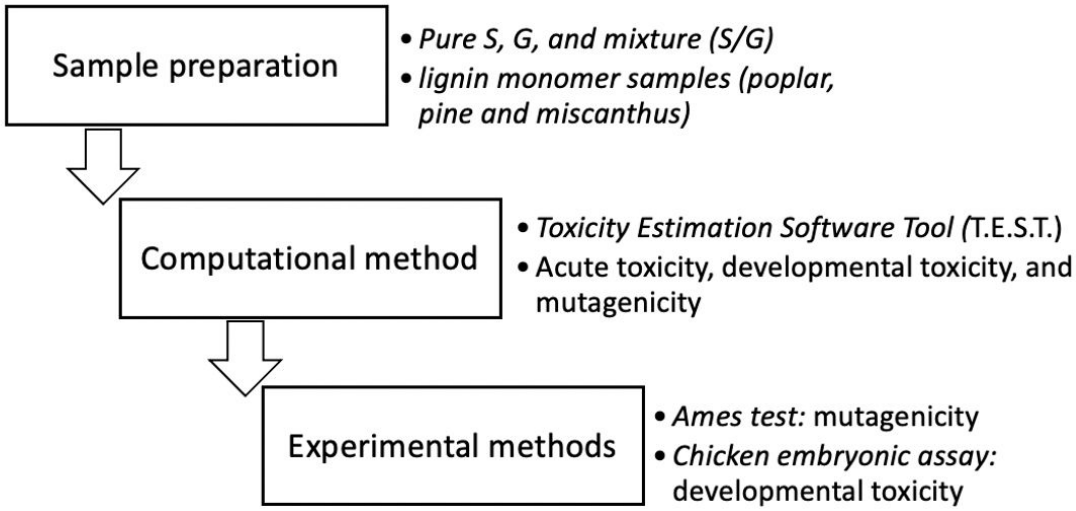

Figure 1 Scheme showing multitiered toxicology evaluation of S/G mixture and lignin monomers

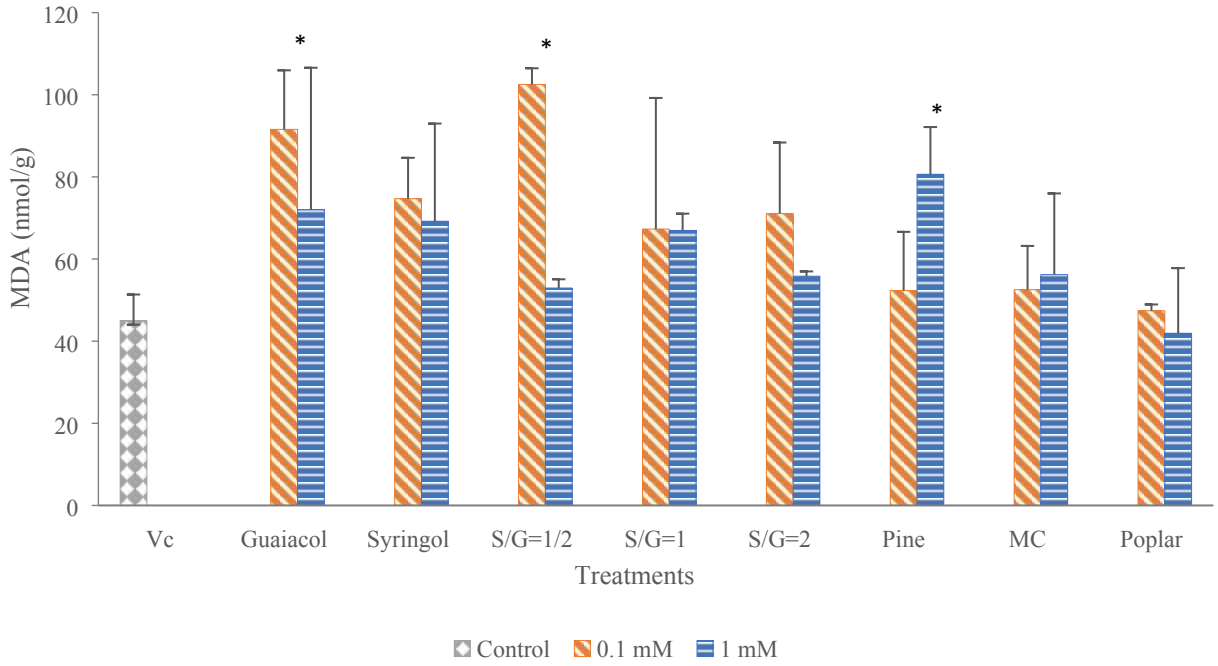

Figure 2 Impacts of Guaiacol, Syringol, three ratios of S/G mixture (S/G=0.2, S/G=1, S/G=2) and three lignin monomers (pine, MC, and poplar) on malondialdehyde (MDA) of livers in chicken embryos. The chemical solutions were at 0.1 mM and 1 mM and injected at 0.2 mL into the egg (average weight 60 g) 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 ± 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