IN SILICO, IN VITRO, AND *IN VIVO* EVALUATION OF THE TOXICITY PROFILE OF NATURAL PHENOLIC COMPOUNDS AND SYNTHESIZED BISPHENOLS

by

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ABSTRACT

Food safety is the priority of the food industry. Natural phenolic compounds are in abundance in plant-based foods, have a wide variety of structures and are known for their potential bioactive benefits; however, the relationship between these biological functionalities and their potential toxicity is not clear. More and more studies have demonstrated potential toxicities of these dietary phenolic components. Bisphenol A (BPA) is an essential building block for many polymeric systems, such as polycarbonates and epoxy resins. Its widespread use in various consumer products and food packaging materials poses significant safety and environmental concerns. Efforts are underway to address the environmental challenges associated with BPA, including regulatory measures and the search for safer alternatives. As society seeks more sustainable, greener and safer alternatives of BPA, it is vital to generate a comprehensive evaluation platform to target its possible toxicity endpoints. The aim of the current project is to investigate the toxicological profile of natural phenolic compounds and synthesized lignin-derivable monomers as BPA alternatives.

In the first study, the developmental toxicity, endocrine disruption effect, and mutagenicity of thymol and carvacrol were investigated at low exposure doses. The results indicated that as phenolic isomers, thymol and carvacrol had different toxicity patterns on the three toxicity endpoints. Carvacrol showed higher binding affinities to two estrogen receptors, had weak estrogenic activity (EA) at 10^{-12} M, and negatively impacted chicken embryonic growth at 50 μg/kg.

In the second study, toxicity of four common flavonoids: genistein, apigenin, quercetin, and luteolin were evaluated and compared. In agreement with the in silico molecular docking results, genistein and apigenin showed higher EA from the MCF-7 cell proliferation assay than EA of luteolin and quercetin. Moreover, genistein and luteolin demonstrated high developmental toxicity in the chicken embryonic assay (at 45–477 μg/kg) with a mortality rate of up to 50%. Among the tested flavonoids, quercetin (a flavonol) with a 2-hydroxyl substitution in the phenol ring exhibited lower developmental toxicity and EA.

In the third study, we investigated the toxicity of two monolignols: guaiacol (G) and syringol (S), mixtures with varied S/G ratio, and three lignin depolymerization samples from poplar, pine, and miscanthus species. The results revealed that the S/G ratio impacts the mutagenicity and developmental toxicity in chicken embryos caused by lignin monomers. The mutagenicity potential of S/G mixtures and lignin monomers was correlated with the syringol proportion, while the adverse effects observed in the chicken embryonic assay were linked to the guaiacol ratio.

In the last three studies (Study 4-6), we focused on exploring the toxicity of bisphenol A (BPA) and lignin-derivable monomers as potential BPA replacements. In the fourth study, genotoxicity of six lignin-derivable bisguaiacols with varying regioisomer contents and degrees of methoxy substitution was investigated. Results showed that most bisguaiacols except m,p'-BGS did neither show signs of mutagenicity in the Ames test nor induce DNA damage in comparison to BPA in the Comet test. The findings suggest that having at least one methoxy ortho to a phenolic hydroxyl group contributed to the lower oxidative DNA damage than BPA.

In the fifth study, the EA and developmental toxicity on chicken embryo model of lignin-derivable bisguaiacols/bissyringols were investigated. Bissyringol A (BSA) with four methoxy groups showed undetectable EA and lack of estrogenic response in the chicken fetal liver. A comparable developmental toxicity was observed from the *in vivo* chicken embryonic assay for lignin-derivable monomers and BPA at environmentally relevant test concentrations. In the sixth study, the *in vitro* metabolism pattern of three lignin-derivable compounds as well as BPA were explored using ultra-performance liquid chromatography-mass spectrometry. Moreover, we conducted the *in vivo* toxicokinetic study of BPA via a chicken embryo model. Our results, in agreement with the predicted data, demonstrated that three lignin-derivable compounds had identical *in vitro* metabolite pathways which are similar to that of BPA.

In summary, we found that the two phenolic monoterpenes and four flavonoids tested in the study demonstrated varied level of EA, mutagenicity, and developmental toxicity depending on their structures at a low exposure range. Moreover, the results showed that the methoxy substituents on lignin-derivable bisphenols appear to be a positive factor to reduce genotoxicity and oxidative DNA damage. The number of methoxy groups on lignin-derivable bisguaiacols/bissyringols plays a role on EA level. Additionally, a novel chicken embryo model was developed to target various critical toxicity endpoints, including developmental toxicity, genotoxicity, endocrine disruption, and metabolism, which were closely related to the structure and treatment dose of the natural phenolic compounds and synthesized bisphenols.

Chapter 1

INTRODUCTION

Natural phenolic compounds refer to a large group of compounds that are present in plants with at least one aromatic ring containing one or more hydroxyl substituents (Bhuyan and Basu 2017). Studies have linked these compounds with promising biological activities, e.g., antioxidant, anti-inflammatory, anticarcinogenic, cardiovascular-protective, and neurotrophic activities (Mak et al. 2006). However, some contradictory results and toxic effects associated with these phenolics have also been reported, including estrogenic activity (EA), cytotoxicity, genotoxicity, and carcinogenic activity (Kyselova 2011; Stich 1991; Resende et al. 2012; Hodek et al. 2006).

Bisphenol A (BPA) is one of the most widely applied synthetic phenolic compounds which is used for the synthesis of an array of polymeric systems. Plastics containing BPA have been used for various applications, including food packaging, pharmaceuticals, dental materials, flame retardants, and other products (Mikołajewska, Stragierowicz, and Gromadzińska 2015). After widespread use of BPA, it has become ubiquitous and humans are exposed to it in daily life (Erler and Novak 2010). In the past two decades, various toxicological problems have been reported on BPA exposure, such as endocrine disruption, reproductive and developmental toxicity, and genotoxicity (Rubin 2011; Møller 2022).

In response to the negative health impacts associated with BPA exposure, a number of bisphenol analogs have been synthesized, including bisphenol F (BPF),

bisphenol S (BPS), bisphenol AF (BPAF), and others, and they are quickly applied as BPA replacements. The products with these commercial bisphenol analogs are usually labeled as "BPA-free" which might mislead consumers to perceive the products as safe. Due to structural similarities and physicochemical properties, most bisphenols have been reported exhibiting similar toxicological profiles with comparable or even stronger toxic potential as BPA. The potential toxicity of natural phenolic compounds and bisphenols are reviewed in Chapter 2.

Recently, lignocellulosic biomass (LB) has emerged as a promising raw material for crafting bio-based polymeric substitutes to traditional petroleum-derived macromolecules (Over et al. 2017). Lignin, as a major component of LB, is a substantial source of natural aromatic chemicals. Lignin-derivable compounds have been regarded as greener and more sustainable materials and are proposed as potential alternatives to commercial bisphenols with a high quality of thermomechanical properties (Bass and Epps, III 2021). It is crucial to understand the potential toxicity of these lignin-derivable monomers in comparison to BPA before applying them in manufacturing processes.

The aim of the current project is to investigate the toxicity of natural phenolic compounds and lignin-derivable monomers on three critical endpoints: endocrine disruption, genotoxicity, and developmental toxicity via a multi-tiered method system including *in silico*, *in vitro*, and *in vivo* models. Ultimately, the goal is to explore the structure-toxicity relationship of phenolic compounds on different toxicity endpoints and to identify safer and greener BPA replacements.

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Chapter 2

LITERATURE REVIEW

2.1 Toxicity studies of natural phenolic compounds

Phenolic compounds are a large class of secondary metabolites that are naturally occurring in plants, vegetables, and fruits. The term 'phenolic compounds' refers to a diverse group of over 8,000 compounds, characterized by possessing at least one aromatic ring with one or more hydroxyl substituents, ranging from simple phenolic molecules to complex polymerized compounds (Bhuyan and Basu 2017). These natural phenolic compounds have been associated with a number of promising biological activities, including antioxidant, anti-inflammatory, anticarcinogenic, cardiovascular-protective, and neurotrophic effects (Zhang et al. 2022). Nevertheless, certain contentious findings and harmful impacts linked to these phenolics have also been documented.

Flavonoids, a widespread group of polyphenolic compounds, constitute one of the most extensive classes among natural phenolic compounds (Mutha, Tatiya, and Surana 2021). Due to the structural similarity of 17β-estradiol, the estrogenic activity (EA) of flavonoids has been reported to occur via interaction with estrogen receptors (ERs), resulting in the disruption of the normal hormone system in humans (Patisaul

and Jefferson 2010; Ye and Shaw 2019). Several studies revealed that isoflavone compounds, such as genistein and daidzein, show estrogenic potency from both an *in vitro* recombinant yeast cell assay and an *in vivo* mice model (Kalita and Milligan 2010; Breinholt et al. 2000). Additionally, luteolin demonstrated potent progesterone antagonist and estrogen agonist activity in *in vitro* assays (Nordeen et al. 2013).

Developmental toxicity is another toxicity endpoint has been associated with flavonoid exposure. A study shows that 15 of 24 tested flavonoids elicited adverse effects on one or more of the developmental or behavioral endpoints on an integrative zebrafish system at exposure levels of 1–50 μM (Bugel, Bonventre, and Tanguay 2016). Another study revealed that kaempferol and quercetin, present in an extract of *Thevetia peruviana* leaves, cause impairment in the reproductive function of female Sprague-Dawley rats by reducing progesterone production (Samanta, Bhattacharya, and Rana 2016). Similarly, fisetin, belonging to the flavonol group, has been reported to interfere with ovarian steroidogenesis and alter the expression of steroidogenic enzymes in granulosa cells (Bujnakova Mlynarcikova and Scsukova 2018).

Furthermore, the pro-oxidant and hepatotoxic activity of natural phenolic compounds has been reported (Galati and O'Brien 2004). An *in vivo* study using the Swiss mice model reported that exposures to high doses of apigenin (100 and 200 mg/kg) led to hepatotoxicity in mice, including increasing levels of malondialdehyde (MDA) and reactive oxygen species (ROS), along with altered gene expression levels related to the oxidative stress and apoptosis (Singh et al. 2012). Furthermore, the

mutagenic activity of quercetin, along with the signs of mutagenicity of luteolin and fisetin, has been reported by using the Ames test (Resende et al. 2012).

2.2 Toxicity studies of bisphenol A

2.2.1 BPA exposure

Bisphenol A (BPA, 2,2-bis(4-hydroxyphenyl)) was first synthesized in 1905 and has been used in the manufacture of polycarbonate plastic and epoxy resin (Rykowska and Wasiak 2006). After been synthesized, the demand and production level of BPA increased due to a number of appealing properties of BPA synthesized plastics, such as high tensile strength, durability, and light weight (Rykowska and Wasiak 2006). Plastics containing BPA have been used for various applications, including food packaging, pharmaceuticals, dental materials, flame retardants, and other supplies (Biau et al. 2007). Following the extensive application of BPA, it is nowadays ubiquitous and humans are exposed to it in daily life (Wetherill et al. 2007). A study documented that BPA (from 0.4 to 149 μg/L) was detected in urine of 92.6% of U.S. participants (n = 2517) (Calafat et al. 2008). In a number of environmental studies, BPA has also been detected in different locations with varied concentrations, including surface-water (Kolpin et al. 2002), effluents (Fernandez, Ikonomou, and Buchanan 2007), groundwater (Barnes et al. 2008), and sediment (Funakoshi and Kasuya 2009; Flint et al. 2012).

2.2.2 Endocrine disruption effect of BPA

BPA has been proven to be an endocrine-disrupting chemical (EDC), acting as an exogenous agent that interferes with the normal function and homeostasis of the hormone system in our body, resulting in various substantial damages and pathological consequences (Vandenberg et al. 2009). The estrogen activity of BPA is one of the critical and widely studied pathways. A number of studies revealed that BPA binds to ERs by competing with the natural estrogen hormones in our body at a low-dose range (about 0.1–1 pM) and alters the related gene expression levels (Alonso-Magdalena et al. 2012). Besides the classical ERs pathway, BPA could also mediate non-ERs dependent pathways by binding to membrane estrogen receptors (mER) (Thomas and Dong 2006). G protein-coupled estrogen receptor (GPER) is one of the most well know mERs and is involved in various pathways causing toxicity (Cariati et al. 2019). In addition, BPA was known to interfere with the androgen pathway with an anti-androgen effect by binding to a androgen receptor (AR) as an antagonist (H. Wang et al. 2017). After binding with AR, the BPA-AR complex was unable to interact with androgen receptor elements, which resulted in the inhibition of gene transcription and the damage to reproductive function and development (Tan et al. 2015). The activation ability of BPA to Peroxisome proliferator-activated receptor (PPAR) gamma and resulting enhanced 3T3-L1 adipocyte differentiation has also been reported (Biasiotto et al. 2016; Ahmed and Atlas 2016). PPARs are known to have a closely relationship with type 2 diabetes, obesity, and other metabolic diseases (Tsuchida et al. 2005). It has been reported that exposure to BPA impairs thyroid

homeostasis and causes thyroid hormone disorders since BPA binds to the thyroid hormone receptor (TR) as an antagonist (Sheng et al. 2012) (Sun et al. 2009). Notably, due to the highly complex and regulatory function of our endocrine system, BPA could disrupt the feedback control system including that of several different endocrine organs, such as the hypothalamic-pituitary-gonadal axis and hypothalamic-pituitary-thyroid axis, which are critically important in neonatal development and metabolism function (Ma et al. 2019).

2.2.3 Genotoxicity of BPA

Genotoxicity is another concern related to BPA exposure. Genotoxic studies of BPA have been extensively conducted primarily through the comet test, chromosome aberrations assay, micronuclei induction method, and γ-H2AX assay. Comet test which detects DNA strand breakage in single cell and oxidative DNA damage by adding specific enzymes (such as formamidopyrimidine DNA glycosylase) has been widely applied in various *in vitro* cell lines and *in vivo* (Møller 2022). The comet test results showed that BPA caused DNA damage in human epithelial type 2 cells (Hep-2) cell at 0.44 nM and oxidative DNA damage in human lung fibroblasts (MRC-5) at 4.4 nM (Ramos et al. 2019). Additionally, previous studies revealed that BPA induced structural chromosomal aberrations in various cell lines, including human peripheral blood mononuclear cells (PBMC) (at 25 – 100 nM) (Di Pietro et al. 2020), Chinese hamster ovary (CHO) cells (80 - 120 μM) (Xin et al. 2015), and human lymphocyte cells (0.88 μM) (Santovito et al. 2018). Recently, measurement of γ-H2AX, an early

stage responding product to DNA double-strand breaks, has been reported as an efficient technique for genotoxic assessment (Rahmanian, Shokrzadeh, and Eskandani 2021). BPA showed a weak genotoxic signal in the γ-H2AX assay on human kidney adenocarcinoma cells (ACHN) at 50 and 100 μM (Audebert et al. 2011).

2.2.4 Developmental and Reproductive toxicity of BPA

The adverse effect of BPA on development and reproduction of different organisms has been well documented. Exposure to BPA across a broad range of concentrations impacted diverse stages of reproductive development, encompassing sex determination and differentiation, gonad maturation, gametogenesis, and gamete quality (Chen et al. 2015). A number of studies show that BPA exposure at environmentally relevant doses during the perinatal period results in a decline in the reproductive capacity of female mice (Cabaton et al. 2011), impact on germ line of male rats (Salian, Doshi, and Vanage 2009), and diminish the spermatogenesis capacity in male mice (Meng et al. 2018). The reproductive disorders in goldfish after the BPA treatment has also been reported, including disrupting ovarian maturation and male germ cell maturation (Wang et al. 2019). Another study revealed that exposing zebrafish to BPA (at 0.228 μg/L) causes female-biased alteration of sex ratio, reduced sperm counts and quality, and male-mediated reproductive failure in their offspring (Chen et al. 2015). Several studies using a zebrafish embryo model showed that BPA exposure at concentrations higher than 500 μg/L induced embryotoxicity and marked

malformations, including pericardial edema, hatching inhibition, decreased heartbeat rate, and spine deformation (Reis et al. 2022; Scopel et al. 2020).

2.2.5 Oxidative stress of BPA

Numerous studies have revealed that BPA can induce oxidative damage, impair antioxidant enzyme activity, and elevate inflammatory cytokines, ROS, and lipid peroxidation in various cell lines and animal models (Tarafdar et al. 2022). Research showed that these changes are closely related to hepatotoxicity, nephrotoxicity, neurotoxicity, and damage in the reproductive system. Hepatotoxicity in BPA-exposed rats (10 – 50 mg/kg) was observed due to increased ROS generation and interruption of the antioxidant system in liver tissue (Hassan et al. 2012). BPA-induced nephrotoxicity has also been reported in a rat model and is attributed to ROS overproduction and mitochondrial dysfunction (Shirani et al. 2019). Additionally, a study using a zebrafish model revealed that BPA causes neurotoxicity (at 500 μg/L) through oxidative stress, inhibiting the activity of catalase (CAT) and superoxide dismutase (SOD), and increasing lipid peroxidation (Gu et al. 2021). In another study, increased ROS levels, inhibition of antioxidant enzyme activity (CAT), and an inflammatory response (IL-1β) were observed in adult male rare minnows with chronic BPA exposure (90 days). These oxidative stress and immune responses might be related to decreased sperm quality, subsequently impairing male reproduction (Zhu et al. 2021).

2.3 Toxicity studies of commercial bisphenol analogs

With more convincing evidence of negative health impacts of BPA, many countries enforce restrictions on its application in food contact materials. Canada was the first country to ban the use of BPA in baby bottles in 2008. Then the European Commission and US Food and Drug Administration (FDA) banned it from use in coatings of infant packaging successively in 2011 and 2013 (Usman & Ahmad, 2019). Therefore, in response to the increased demand for BPA-free products, a variety of BPA alternative compounds has emerged, including Bisphenol AF (BPAF), Bisphenol F (BPF), and Bisphenol S (BPS). These BPA alternatives typically possess competitive thermal properties and can be utilized in the production of polymer-based materials. However, due to structural similarities and physicochemical properties, it can be hypothesized that these alternatives might exhibit similar toxicological profiles, with comparable or even stronger toxic potential than BPA.

It has been reported that EA has been detected in the four extract solutions of BPA-free thermoplastic resins by MCF-7 and BG1Luc assays (Bittner, Denison, Yang, Stoner, & He, 2014). The EA of two leading alternatives, BPS and BPF, has been reported, and they can also agonize the thyroid hormone signaling in the thyroid receptor (TR)-mediated luciferase reporter gene assay and promote GH3 cell proliferation (Zhang et al., 2018). One recent study revealed that the bisphenol analogues (BPB, BPF, and BPS) disrupted reproductive functions and impaired ovarian development in adult female rats (Ijaz, Ullah, Shaheen, & Jahan, 2020). In another study, BPAF and BPS showed higher developmental toxicity than BPA in a

chicken embryo model, adversely impacting development, growth, and survival in a dose-dependent manner at a low concentration range of 3 to 30 nM (Harnett et al. 2021). Additionally, oxidative damage and inflammation effect were observed in both BPF and BPS exposed zebrafish intestine (Wang et al. 2021) BPS has been reported to induce hepatotoxicity in a mice model at 5000 μg/kg by oral administration, relating to the interference in the oxidative system (Zhang et al. 2018). BPF and BPAF showed higher genotoxic concern than BPA inducing DNA double strand breaks by γ-H2AX assay (Hercog et al. 2019).

2.4 Metabolic reactions of bisphenol compounds

The metabolic reaction in the human body is important when assessing the toxicity of these bisphenol compounds since they are not persistent in their origin forms but rather go through metabolism (Skledar and Mašič 2018). Moreover, these metabolic transformations of chemicals will affect the toxicity response such as endocrine activities. In humans, the major metabolization of bisphenols is through conjugation reactions with glucuronic acid and sulfate (Gramec Skledar et al. 2015). The effects of their metabolism on toxicity and the endocrine activity need to be assessed to better understand the toxic responses of the BPA replacements.

BPA is metabolized primarily *in vivo* by the metabolic phase II group of enzymes UDP-glucuronosyltransferase (UGTs) in the intestine and liver into BPA-glucuronide (BPA-G), which is also referred to as a detoxification pathway, generating the BPA glucuronide. This major metabolite, the glucuronide of BPA isolated from

adult female F-344 and CD rats, has been reported with no estrogenic activity (Snyder et al. 2000). In the rat model, the major metabolite of BPA in urine and plasma is the monoglucuronide conjugate. Similarly, under the *in vitro* incubation condition, BPA glucuronide could be found as the major metabolite generated by the UGT2B1 enzyme in rat liver microsomes and by human recombinant UGT isoforms (Pottenger et al. 2000). Moreover, the BPA oxidative metabolite has been reported in an *in vitro* metabolism study by cytochrome P450s. The 4-methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene (MBP) is one of the BPA oxidative products, which showed about 500-fold higher estrogenic activity than BPA (Skledar and Mašič 2016). However, the oxidative metabolites of BPA were mainly reported for *in vitro* studies, since BPA glucuronidation is a much faster and prevalent metabolic reaction *in vivo* (Skledar and Mašič 2018). BPS is one widely used BPA alternative. As reported in a human study, BPS is predominantly metabolized by the conjugation reactions and that 97% of the BPS in human urine was detected as the conjugated form. BPF sulfate is the main metabolite found both in vivo female Sprague-Dawley rats and *in vitro* study using the HepG2 cell line (N. Cabaton et al. 2006; Dumont et al. 2011).

2.5 Utilization of the chicken embryo model in toxicity studies

The *in vivo* animal model, including mice, rats, and monkeys, has long been pivotal in the field of toxicology, regarded as a "gold standard" for evaluating human risk (Fielden and Kolaja 2008). Researchers have emphasized the '3R' strategy—Replacement, Reduction, and Refinement—in toxicity experiments (Ghimire et al.

2022). This includes exploring alternative methods to decrease the use of animals, substituting them with *in vitro* models, and adhering to animal welfare policies. The chicken embryonic model has been reported as being a suitable alternative embryo model to traditional rodent animals in investigating toxicity of EDCs (Kue et al. 2015; Haseena Bhanu SK 2014; Ghimire et al. 2022). As a cost-effective and fast-turnout embryological model, the chicken embryo has also been reported to be more sensitive to a wide variety of contaminants and chemicals. The chicken genome has been fully sequenced and annotated which enables researchers to understand the underlying mechanisms in toxicological studies (Ribatti 2017). Disruption in reproductive organ development and alteration of hormone-responsive gene expression during chicken embryogenesis have been reported after exposure to different EDCs (Mentor et al. 2020; Jessl et al. 2018; Crump, Chiu, and Williams 2016). Additionally, developmental toxicity and genotoxicity of EDCs have been revealed using a chicken embryo model (Crump, Chiu, and Williams 2018; Uggini and Suresh 2013).

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Chapter 3

CHICKEN EMBRYONIC TOXICITY AND POTENTIAL IN VITRO ESTROGENIC AND MUTAGENIC ACTIVITY OF CARVACROL AND THYMOL IN LOW DOSE/CONCENTRATION

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3.1 Abstract

Thymol and carvacrol are phenolic isomers with the potential for developmental toxicity and endocrine disruptions (ED) at low concentrations. However, few reports estimated their toxicity and ED below 10^{-6} M (150 μg/L) (MW of thymol and carvacrol: 150 g/mol). In this study, both chemicals were determined for the developmental toxicity and potential ED at 500 μg/kg and 50 μg/kg using the chicken embryonic assay, potential estrogenic activity (EA) at 10^{-12} to 10^{-7} M (1.5×10^{-4} to 15 μg/L) by the MCF-7 cell proliferation assay, mutagenicity at 10^{-12} to 10^{-6} M (1.5×10^{-4} to 150 μg/L) by the Ames test, and an in silico method for ED. Carvacrol showed mutagenic risks at 10^{-7}, 10^{-8}, and 10^{-11} M (15, 1.5, and 0.0015 μg/L) while thymol at 10^{-6} and 10^{-8} M (150 and 1.5 μg/L). Carvacrol negatively impacted embryonic growth at 50 μg/kg, with weak EA at 10^{-8} M (1.5 μg/L). Carvacrol but not thymol had weak EA at 10^{-12} M (1.5×10^{-4} μg/L). Molecular docking to 14 types of hormone-related receptors revealed that carvacrol had higher binding affinities to two estrogen receptors and the mineralocorticoid receptor than those to thymol. Carvacrol

and thymol varied in toxicities due to a different location of one phenol hydroxyl group.

KEYWORDS: Thymol, Carvacrol, Developmental toxicity, Mutagenic activity, Estrogenic activity, Molecular docking

3.2 Introduction

Essential oils are obtained from plant materials, and have been traditionally used in food products, medicine, and perfume industries due to their unique flavors, antioxidant, and antimicrobial properties (Bakkali et al. 2008). Carvacrol and thymol are two major constituents present in thyme and oregano essential oils. These two compounds are phenolic isomers that exhibit significant antibacterial and antioxidant activities (Lambert et al. 2001). The chemical structures of the two chemicals are just different in one position of a phenolic hydroxyl group, as shown in Figure 3.1.

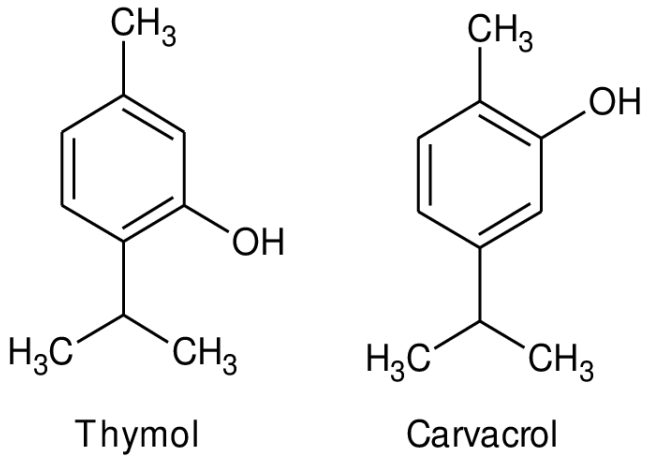


Figure 3.1: Structures of thymol and carvacrol

Thymol, carvacrol, and thyme oil have been approved by the Food and Drug Administration (FDA) as a direct food additive in food for human consumption (Lee et al. 2003). They are also registered flavorings and foodstuffs by the Council of Europe (Council of Europe, 2000) and the Food and Agriculture Organization/World Health Organization (FAO/WHO). These natural compounds have been used as food additives at low concentrations (2.5-150 mg/kg wet weight) to improve food quality. Levels of thymol vary in different foods; the concentrations are reported as 44 mg/kg in ice cream, 2.5 to 11 mg/kg in non-alcoholic beverages, 9.4 mg/kg in candy, 5 to 6.5 mg/kg in baked goods, and 100 mg/kg in chewing gum (Fenaroli 1975). For carvacrol, similar levels are also added to foods: 16 mg/kg in baked goods, 28 mg/kg in non-alcoholic beverage, and 8 mg/kg in chewing gum (Azirak and Rencuzogullari 2008). Moreover, these natural compounds can be used in food packaging at a relatively higher dose (50-100 g/kg) (Cerisuelo et al. 2012). Thymol and carvacrol are used as active agents due to their antimicrobial or antioxidant activities and incorporated in the food packaging to be effectively released to the food surface at a controlled rate (Cerisuelo et al. 2012). The antimicrobial capacity of plastic films was increased by coating microcapsules containing carvacrol and thymol when used in fresh food preservation (Guarda et al. 2011). Thymol and carvacrol might be migrated into food from the packaging materials to induce low exposure levels in humans.

More studies are currently conducted to determine the adverse health effects of endocrine disrupting chemicals (EDC), which are compounds affecting the natural balance of hormones in humans and animals. Public and scientific concerns about

potential EDC on human and animal health have risen in recent years (Gavaric et al. 2015). For example, bisphenol A (BPA), a widely used monomer of polycarbonate plastics, has demonstrated estrogenic activity (EA) by in vitro and in vivo experiments, even it displays lower binding affinities for estrogen receptor (ER) α and ERβ compared to 17-β estradiol (E2) (Shanle and Xu 2011). BPA and its analogs cause adverse health effects in human and wild animals (Peng, Nicastro, Epps, & Wu, 2018; Vandenberg 2014; Pelch et al. 2017), and their EA could be associated with the non-classical estrogen triggered pathways like non-nuclear initiation mechanisms (Alonso-Magdalena et al., 2012). Furthermore, besides two estrogen receptors (ERs), other cellular targets also play roles in the EA of BPA, such as the estrogen-related receptor γ (ERRγ) (Okada et al. 2008; Delfosse et al., 2012). Since the thymol and carvacrol share a similar structure motif with E2, it is highly possible that both chemicals have EA and interfere with estrogen signaling by interacting with ERs directly or indirectly. Due to the complex biology in toxicity and endocrine disruption (ED), it is a big challenge to fully evaluate the safety of these natural compounds. It is recommended by the guidelines of the U.S. FDA and National Institute of Environmental Health Sciences that a multitiered approach including both in vitro and in vivo methods is a better tool to assess the toxicity and ED properties of the potential EDCs (Wu and Peng 2018). This multitiered approach is developed in our current study to evaluate the toxicity and ED of thymol and carvacrol. We used the MCF-7 cell proliferation assay, an *in vitro* bioassays, to determine the proliferation of MCF-7 human breast cancer cells as the endpoint for quantifying whole-cell-level EA. EA

evaluates whether the chemicals mimic the actions of naturally occurring estrogens and is one of the most studied ED. Moreover, we used a chicken embryo model to test the potential ED and development toxicity in vivo because the embryos have emerged as a reliable model for testing toxicants and environmental pollutants (Biau et al. 2007). To determine the carcinogenicity and mutagenicity, which are the key toxicological effects with the deepest concern for human health, we used the Ames test (Resende et al. 2012), in the presence and absence of in vitro metabolizing systems.

One innovative design of our study is the test concentration ranges, with 500 μg/kg and 50 μg/kg used in the chicken embryonic assay, 10^{-12} to 10^{-7} M (1.5×10^{-4} to 15 μg/L) for the MCF-7 bioassay, and 10^{-12} to 10^{-6} M (1.5×10^{-4} to 150 μg/L) for the Ames test. We tested such low concentrations of both chemicals because many previous toxicity studies about these natural compounds are focused on relatively higher concentrations such as 110–233.3 mg/kg in mice (intraperitoneal) (Suntres, Coccimiglio, and Alipour 2015). However, low exposure might be present as currently limited knowledge is available regarding the human exposure level of both chemicals. According to studies performed in piglets, almost complete absorption of carvacrol and thymol was determined in the stomach and the proximal small intestine, indicating a cumulative absorption of more than 90% (Michiels et al. 2008). This high absorption of both chemicals makes it important to determine their toxicity and ED even at a low exposure level. If the levels of carvacrol or thymol in food are 100 mg/kg, then the human exposure level to these natural compounds is estimated at 0.1818 mg/kg for an

adult (average body weight of 55 kg) who consume 100 g of carvacrol- or thymol-containing food and assumptively fully absorb the natural compounds. Considering the human equivalent dose factor from chicken (×18.5) (ATSDR. 2011) and the human safety factor (÷10) (Nair and Jacob 2016), the lower dose (50 μg/kg) and the higher dose (500 μg/kg) of thymol and carvacrol exposure level used in the chicken embryonic assay, can be converted to the human equivalent doses as 92.5 and 925 μg/kg ($\frac{50\times18.5}{10}$=92.5 μg/kg; $\frac{500\times18.5}{10}$=925 μg/kg). In addition, it is necessary to assess the toxicity and EA at low concentrations (around 1×10^{-3} μg/kg) of thymol and carvacrol when considering the presence of non-monotonic dose response (NMDR) in EDCs (Figure 3.2). This is the main reason to determine thymol and carvacrol at 10^{-12} to 10^{-6} M (1.5×10^{-4} to 150 μg/L) using the MCF-7 bioassay and Ames test. The slope of the response curve will change, meaning the lower dose might show a higher effect than that of the higher dose, so a safe dose determined from high dose does not guarantee safety at the low dose, and effects from the low dose should be determined (Figure 3.2, Vandenberg et al. 2012, 2014). To assess the binding affinity between thymol and carvacrol to 14 types of hormone-related nuclear receptors, we used an in *silico* molecular docking method. This structure-based simulation can help to reveal the mechanisms of the EDC potential of thymol and carvacrol and provide insights for further study of the toxicity of these natural compounds.

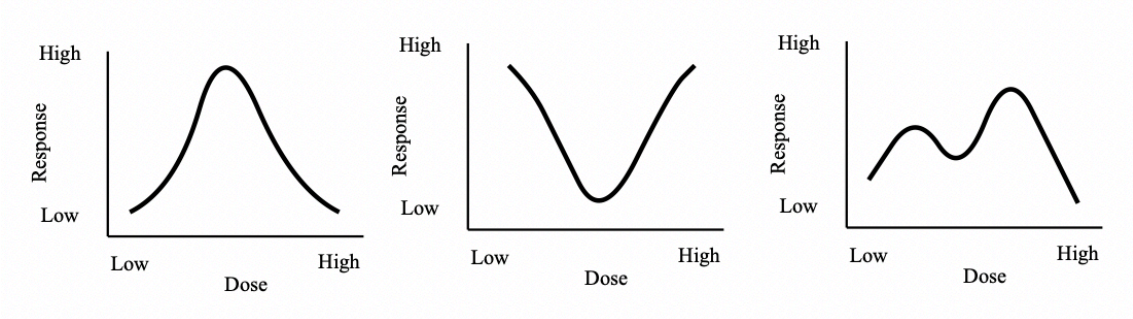


Figure 3.2: Three different types of NMDRCs including an inverted U-shaped curve, a U-shaped curve, and a multiphasic curve (Vandenberg et al., 2012).

3.3 Materials and methods

3.3.1 Chemicals and cell lines

17β-estradiol (E2), Bisphenol A, 17 beta-Estradiol, thymol (99.6%), and carvacrol (99.5%) were purchased from Fisher Scientific. MCF-7 cells were purchased from American Type Culture Collection (ATCC No. HTB-22). Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Cat. No.: 12-430-054), phenol red-free DMEM (Gibco, 21-063-029), fetal bovine serum (FBS) (Gibco, 16-140-071), charcoal-stripped FBS (Gibco, 12-676-029), penicillin-streptomycin (Gibco, 15-140-148), dimethyl sulfoxide (DMSO) (D1391) and phosphate buffered solution (PBS) (Gibco, 20-012-027) were purchased from the Fisher Scientific website, along with the cell culture used 96-Well Clear Polypropylene Microplates (Corning, 05-539-200) and polystyrene T-25 flasks (Corning, 08-772-45).

3.3.2 Chicken embryonic assay

In total (three independent trials), 108 Leghorn eggs were obtained from the University of Delaware research farm, and the eggs were randomly assigned at day 0 to each treatment and control group (total 9 groups and 12 eggs for each group). Only 90 out of 108 eggs were fertilized and used for the chemical injection. The eggs were weighed and labeled for each treatment. On day 7, the eggs were candled to locate the air cell, and a suitable location marked for injection. A hole was drilled at the marked location, and 0.2 mL of vehicle control (VC) or chemical solution (0.1 mM and 0.01 mM for E2 and BPA, 1 and 0.1 mM for thymol and carvacrol) was injected into the egg (average weight 60 g) yielding a final dose of 91 and 9.1 μg E2/kg, 76.7 and 7.67 μg BPA/kg, 500 and 50 μg thymol/kg, and 500 and 50 μg carvacrol/kg of egg. The corresponding human equivalent doses can be obtained by the dose conversion equation (1). These concentrations were chosen based on our preliminary research and a previous report, which showed chemical effects on chicken embryos without generating precipitation of these compounds (Crump, Chiu, and Williams 2016). In our preliminary experiment, we tested the effects of thymol and carvacrol from 5 to 500 μg/kg in the chicken embryo model. There were no dead and deformed embryos, and no significant changes in the other phenotypes for the 5 μg/kg treatment from the 6 embryos, so we chose the two higher dosages (50 and 500 μg/kg) in the current research in the manuscript. In addition, these two dosages are correlated well to the human exposure levels of thymol and carvacrol. The hole was sealed with Duco Cement, and eggs were put back in a 38°C and 60% humidity incubator.

$$human\ equivalent\ dose = \frac{chemical\ dose\ in\ chicken\ embryo \times 18.5}{10} \quad (1)$$

In which 10 is the human safety factor, 18.5 is the dose conversion factor from chicken to human (ATSDR., 2011; Nair and Jacob 2016).

On day 18, all eggs were placed in the refrigerator at 4°C overnight to euthanize the embryos and then opened on day 19. All embryos were dissected to observe any abnormality of organ development, and the following measurements were recorded: malformation, embryo mass, liver mass, and heart mass. The liver somatic index (LSI) was calculated as LSI = liver mass/embryo mass × 100%. LSI is considered a general indicator of health and is responsive to environmental contaminant exposure. The liver samples were collected for lipid peroxidation measurement. The liver samples were firstly placed on ice and homogenized with the buffer to prepare liver tissue homogenates.

Malondialdehyde (MDA) levels were then measured on the liver tissue homogenates, following the protocol of TBARS Assay Kit (Cayman Chemical, MI USA), and calculated as an index of lipid peroxidation. MDA reacts with thiobarbituric acid (TBA) as a TBA reactive substance (TBARS) to produce a red-colored complex which has peak absorbance at 530 nm. Aliquot of 3 mL phosphoric acid (1%) and 1mL TBA (0.6%) was added to 0.5 mL of liver homogenate in a centrifuge tube, and the mixture was heated for 45 min in a boiling water bath. After cooling, 4 mL of n-butanol was added to the mixture and vortex-mixed for 1 min, followed by centrifugation at 20,000 rpm for 20 min. The organic layer was

transferred to a fresh tube, and its absorbance was measured at 530 nm and compared with values obtained from MDA standards. The kit provided 500 μM stock malondialdehyde for the standard curves. The results were expressed as nmol of MDA g^{-1} liver. Each test was repeated in three independent trials and in duplicate for each trial.

3.3.3 MCF-7 Cell Proliferation Assay

MCF-7 cells were grown and maintained in polystyrene T-25 flasks (Corning, Inc.) as detailed in our previous paper (Peng et al., 2018; Peng et al. 2020). Cell proliferation assay was measured by MTT assay in a microplate reader (Synergy 2, Bio-Tek, instruments, Winooski, VT) as described in Peng et al (2018). Briefly, MCF-7 cells were maintained in phenol red DMEM with 10% FBS, 1% PS, and 0.25% Insulin in 25 cm^2 plastic flasks (Corning, Inc.) at 37 °C in a 5% CO_2 humidified atmosphere. Cells were sub-cultured when they reached about 80% confluency using pre-warmed trypsin to digest adherent cells. MCF-7 cells were seeded into 96-well polystyrene plates at 3500 cells/well density with EA free culture medium containing phenol red-free DMEM, 5% charcoal-stripped FBS, 1% PS, and 0.25% Insulin. After 24 hours, cells were treated with fresh EA free medium containing the test chemicals (E2, thymol, and carvacrol) at six different concentrations ranged from 10^{-12} to 10^{-7} M (1.5×10^{-4} to 15 μg/L). The EA of test chemicals was shown as the relative maximum %E2 (%RME2) calculated from the following equation (2):

$$100 \times (\text{OD of test} - \text{OD of VC})/(\text{MAX OD of E2} - \text{OD of VC}) \quad (2)$$

The cells were treated with the test chemicals for a total of 6 days, and the chemical containing medium was refreshed every 2 days. An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was added to each well. All supernatants were carefully removed after 4 h incubation and then the DMSO was added to dissolve crystals. The cell proliferation rate was quantified by measuring the absorbance at 570 nm using a microplate reader (BioTek Synergy ^{2}). Each test was repeated in three independent trials and in triplicate for each trial.

3.3.4 Ames test

The Ames test was conducted using the Salmonella typhimurium tester strains TA 98, TA 100, and TA 102, purchased from Molecular Toxicology Inc (Boone, NC, USA) with a preincubation method as described by Maron & Ames (1983). 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. The metabolic activation mixture (S9) from livers of Sprague–Dawley rats was freshly prepared before each test. Each test compound was first dissolved in DMSO and then diluted by PBS buffer to reach the concentration from 10^{-12} to 10^{-6} M (1.5×10^{-4} to 150 μg/L). The 0.05 mL of tested compounds were added to a 0.5 mL of S9 (or 0.5 mL PBS in without S9 mixture) and 0.1 mL of bacterial culture, and the mixture incubated at 37 °C for 20 – 30 min. Then the 2 mL of top agar was added, and the mixture poured onto a plate containing the minimal agar. After 48 h incubation, the His+

revertant colonies on plates were counted manually. Each test was repeated in three independent trials and in duplicate for each trial.

3.3.5 Molecular Docking

The potential endocrine disrupting nature of thymol and carvacrol was systematically assessed on the docking Interface for Target Systems (DoTS) platform (named endocrine disruptome tool) via AutoDock Vina (Kolšek et al. 2014; Devillers, Bro, and Millot 2015). The ligand structures were generated by ChemSketch to obtain the SMILES (Simplified Molecular-Input Line Entry-System) files as input for the endocrine disruptome tool. The binding affinity between three ligands (E2, thymol, and carvacrol) and 14 nuclear receptors (androgen receptor (AR), estrogen receptors α (ER α) and β (ER β), glucocorticoid receptor (GR), liver X receptor (LXRα, LXRβ), mineralocorticoid receptor (MR), progesterone receptor (PR), thyroid receptor (TRα, TRβ), peroxisome proliferator-activated receptors (PPAR α, PPARβ, PPAR γ), Retinoid X receptor (RXR) and 4 antagonist conformations (AR an, ER α an, ER β an, GR an)) was stimulated in the docking. The four probability binding classes are color-coded as follows: red indicates a high level of binding, orange indicates good binding, yellow indicates medium binding, and green indicates low binding.

3.3.6 Data analysis

The results were analyzed with the statistical software package JMP (JMP PRO 13). In the chicken embryonic assay, the morphological and developmental

endpoints (ratio of the embryo to egg weight, the liver somatic index, the weight of embryo and organs of the chicken embryo), and the lipid oxidation among groups, were evaluated by a one-way analysis of variance (ANOVA) followed by the Tukey's test for multiple comparisons among all treatments and control groups (JMP PRO 13). Similarly, in the MCF-7 cell proliferation assay, the cell proliferation rates were analyzed by one-way ANOVA and followed by the Tukey's test to compare thymol, carvacrol, and E2's findings between each concentration (JMP PRO 13). In addition, one-way ANOVA followed by Dunnett's test was used in the Ames test to compare the revertant numbers of treatment groups to those of VC (JMP PRO 13). Changes were considered statistically significant if $p < 0.05$. The mutagenic index (MI) was also calculated for each concentration using the mean number of revertants per plate treated with the test compound divided by the mean number of revertants per plate treated with the negative (solvent) control. A test compound was considered mutagenic when a dose-response relationship was detected, and a two-fold increase in the number of mutants ($MI \geq 2$) was observed in at least one concentration. If only one of these two criteria was met, the sample was considered to present signs of mutagenicity (Resende et al. 2012).

3.4 Results

3.4.1 Different in vivo effects of thymol and carvacrol in chick embryos

The eggs were randomly assigned at day 0 to each treatment and control group (12 eggs for each group). On the injection day (day 7), the 18 unfertilized eggs were removed and not recorded in Table 3.1. In addition, if the embryos died within the first two days post injection, they were due to inappropriate operation such as higher injection speed or less vertical angle, so the embryo death within two days was not recorded in Table 3.1 (one in 91 μg E2/kg group and the other in the 76.7 μg BPA/kg group). Both scenarios resulted in a different number of total fertilized eggs for some chemical used in Table 3.1. A smaller number of chicken embryos were used for a higher dose of E2 group due to the removal of 5 unfertilized eggs and one dead embryo within the first two days post-injection. The mortality rate of this group was at 50%, with 3 dead out of 6 embryos during the embryonic development. It agreed well with the average mortality rate calculated from the accumulated data of the E2 treatment (91 μg/kg, 12 deaths out of 22 embryos, 54.5%) in all our past toxicity studies using the E2 as a positive control up to the project time (the six embryos used in this study not included in these 22 embryos). The eggs were candled every two days after the chemical injection on day 7 of the chicken embryonic stage. The death of embryos that occurred after the first two days were caused by the chemicals and recorded as death rate shown in Table 3.1. There were no dead or deformed embryos in the solvent control group, which meant the solvent used for dissolving each

chemical was safe for chicken embryos. The higher dose (91 μg/kg) of E2 had the highest mortality rate (50%) and was followed by the lower dose of carvacrol (50 μg/kg) group with 45.5% mortality (Table 3.1). In contrast to the higher mortality rate in higher test doses in traditional toxicity tests, carvacrol at 500 μg/kg only had 12.5% mortality, less than the rate observed at 50 μg/kg group which indicated the potential NMDR for carvacrol. The two doses of BPA treatments had mortality rates at 27.3% and 12.5%, respectively. Compared with the finding from carvacrol (Table 3.1), thymol did not induce any embryo death at two test dosages. In addition, three different types of malformations were observed for three treatment groups. There was no malformation in the solvent group, while 1 of 12 embryos in the lower dose of E2 treatment group showed head deformities (acrania), and 2 of 11 in the lower dose of BPA group showed stunting. Malformation of curled claw and everted viscera was observed in embryos receiving higher dose of thymol injection (12.5%). None of the malformation was observed in carvacrol treatment groups.

Table 3.1: Mortality rate and malformation rate of chicken embryos treated with different chemicals.

treatment	solvent control (1% DMSO)	E2		BPA		Thymol		Carvacrol	
		Chemical solution concentration (mM)							
		0.01	0.1	0.01	0.1	0.1	1	0.1	1
		Treatment dose in egg (μg/kg)							
		9.1	91	7.67	76.7	50	500	50	500
∑ fertilized eggs	12	12	6	11	8	12	8	11	8
mortality rate	0.0% (0)	16.7% (2)	50% (3)	27.3% (3)	12.5% (1)	0.0% (0)	0.0% (0)	45.5% (5)	12.5% (1)

malformation rate	0.0% (0)	8.3% (1)	0.0% (0)	18.2% (2)	0.0% (0)	0.0% (0)	12.5% (1)	0.0% (0)	0.0% (0)

The data are presented as a total number of observations from three independent trials. The number in parentheses represented the number of dead chicken embryos or malformed chicken embryos. The chemical solution (0.1 mM and 0.01 mM for E2 and BPA, 1 and 0.1 mM for thymol and carvacrol) was injected at 0.2 mL into the egg (average weight 60 g) yielding a final dose in egg below: 91 μg E2/kg, 9.1 μg E2/kg, 76.7 μg BPA/kg, 7.67 μg BPA/kg, 500 μg thymol/kg, 50 μg thymol/kg, 500 μg carvacrol/kg, 50 μg carvacrol/kg.

For the ratio of embryo weight to egg weight, the solvent control group had the highest value at 0.42 compared with others (Table 3.2). The lower dose of carvacrol (50 μg/kg) significantly decreased this ratio ($p < 0.05$), which indicated that it harmed the normal development of chicken embryos. No significant decrease was found in other treatment groups. A significant difference was observed between the two doses of carvacrol treatments ($p < 0.05$). The lower value in the lower dose of the carvacrol group suggested that a lower dose of carvacrol might have more adverse effects on chicken embryo development compared with its higher dose treatment. This finding again indicated the potential NMDR for carvacrol, as shown in finding related to the mortality rate.

Table 3.2: The ratio of embryo to egg weight, liver somatic index (%), and weights of chicken embryos and major organs impacted by injection of E2, BPA, thymol, and carvacrol solutions.

Groups	Injection dose (mM)	Final dose (μg/kg)	Ratio of embryo to egg weight	Liver somatic index (%)	Weights (g)			
					Egg	Embryo	Liver	Heart
Solvent control	1% DMSO	N/A	0.42 ± 0.012	2.18 ± 0.32	55.15 ± 5.08	23.16 ± 0.12	0.50 ± 0.04	0.18 ± 0.02
E2	0.01	9.1	0.39 ± 0.035	2.28 ± 0.24	58.64 ± 1.54	22.87 ± 0.04	0.48 ± 0.06	0.19 ± 0.01
	0.1	91	0.37 ± 0.035	1.78 ± 0.23	55.95 ± 5.12	20.70 ± 0.12	0.42 ± 0.08	0.19 ± 0.01
BPA	0.01	7.7	0.33 ± 0.028*	1.99 ± 0.10	62.06 ± 2.69	20.48 ± 0.02	0.41 ± 0.05	0.20 ± 0.01
	0.1	76.7	0.38 ± 0.023	1.95 ± 0.21	57.33 ± 4.06	21.78 ± 0.45	0.49 ± 0.10	0.21 ± 0.00
Thymol	0.1	50	0.3 8± 0.049	2.14 ± 0.23	56.21 ± 4.67	21.36 ± 0.23	0.48 ± 0.10	0.18 ± 0.04
	1	500	0.38 ± 0.014	2.03 ± 0.38	58.79 ± 2.08	22.34 ± 0.07	0.48 ± 0.06	0.18 ± 0.03
Carvacrol	0.1	50	0.34 ± 0.007*#	2.48 ± 0.30#	67.68 ± 6.33	23.01 ± 0.04	0.53 ± 0.04#	0.18 ± 0.02
	1	500	0.40 ± 0.014	1.81 ± 0.04	52.19 ± 2.55	20.87 ± 0.12	0.43 ± 0.03	0.19 ± 0.00

All values are expressed as mean ± standard deviation (SD) from three independent trials. Differences were evaluated using ANOVA followed by the Tukey's test, and statistical significance was indicated by $p < 0.05$. * means a statistically significant difference compared to vehicle control; # means a statistically significant difference within two doses of the same chemical treatment group.

Moreover, as a general indicator of health in response to environmental contaminant exposure, the LSI values are shown in Table 3.2. In this study, no significant difference in LSI levels was observed between treatment groups and the solvent control, even though a 16.9% decrease was determined in the higher dose of

carvacrol (500 μg/kg) when compared with the data from the solvent control. A significant difference only was observed between the two doses of carvacrol treatments ($p < 0.05$), and the lower dose of carvacrol treatment had a higher LSI than the LSI in the higher dose treatment. The heart and liver are two major and important organs in chicken embryos. Treatments of lower dose of BPA, higher dose of E2 and carvacrol resulted in lower liver weight, with 0.41 ± 0.05 g, 0.42 ± 0.08 g, and 0.43 ± 0.03 g (Table 3.2), respectively. All the other groups had comparable liver weight as that of the control group at 0.50 ± 0.04 g. There was no significant difference in the heart and liver weights between each treatment group and solvent control ($p > 0.05$). Significantly lower liver weight was detected in the higher dose of carvacrol group (500 μg/kg) than the low dose one ($p < 0.05$). The heart weight of each group had a similar value at 0.19 g (Table 3.2).

As a naturally occurring product of lipid peroxidation, hepatic MDA level is indicative of oxidative stress. To evaluate the oxidative damage caused by thymol and carvacrol injection in vivo, MDA level in chicken liver tissues was determined and is shown in Figure 3.3. The control group had the lowest value of MDA at 60.37 nmol/g, and all other treatment groups had a higher level of MDA ranging from 81.26 to 114.83 nmol/g. Compared to the solvent control group, a significant increase ($p < 0.05$) of MDA values were found in the higher dose of BPA, along with the lower dose of E2 and carvacrol (50 μg/kg). Moreover, no statistically significant difference was found between the two doses of thymol (or carvacrol) groups, and both of them did not have significantly higher MDA when compared with E2 or BPA groups.

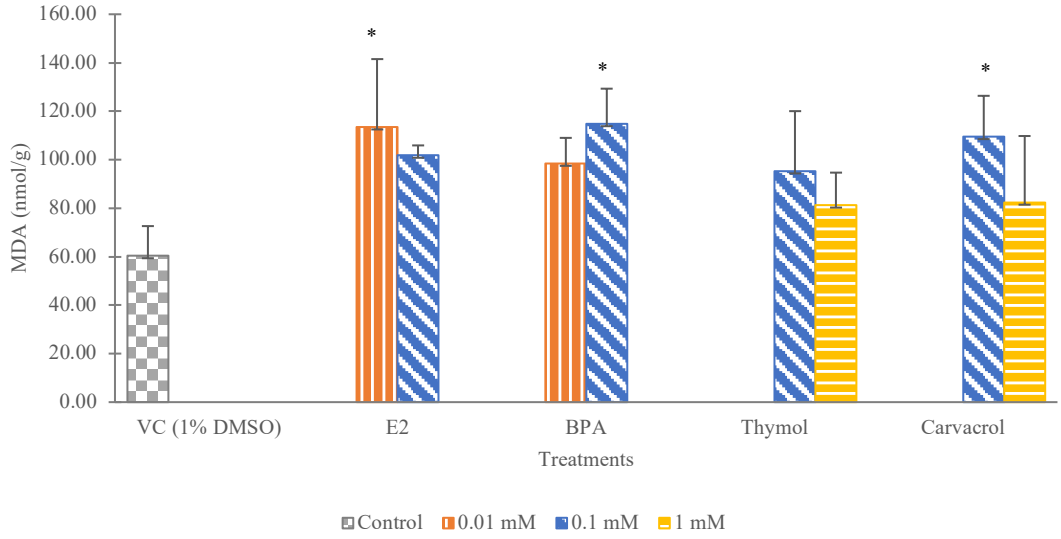


Figure 3.3: Impacts of E2, BPA, thymol, and carvacrol on MDA value.

All values are expressed as mean ± standard deviation from three independent trials. Differences were evaluated using ANOVA followed by the Tukey's test and statistical significance was indicated by $*p < 0.05$ (or $** < 0.01$) compared to vehicle control (VC). The chemical solution (0.1 mM and 0.01 mM for E2 and BPA, 1 and 0.1 mM for thymol and carvacrol) was injected at 0.2 mL into the egg (average weight 60 g) yielding a final dose in egg below: 91 µg E2/kg, 9.1 µg E2/kg , 76.7 µg BPA/kg, 7.67 µg BPA /kg, 500 µg thymol/kg, 50 µg thymol/kg, 500 µg carvacrol/kg, 50 µg carvacrol/kg.

3.4.2 Thymol and carvacrol had different EA when tested by MCF-7 cell proliferation assay

To investigate the potential EA, MCF-7 cells were exposed to increasing concentrations of E2, thymol, and carvacrol between 10^{-12} and 10^{-7} M (concentration range from 2.7×10^{-4} to 27 μg/L for E2, 1.5×10^{-4} to 15 μg/L for thymol and carvacrol). The chemicals were considered to have undetectable EA levels when the absolute %RME2 less than VC + 3 SD (Yang et al., 2014). In our study, we calculated the VC+3 SD in Figure 3.4A was 30%, and in Figure 3.4B was 21%. In this assay, E2 served as the positive control and the maximum EA of E2 was obtained at 10^{-8} M (1.5 μg/L).

As shown in Figure 3.4A, thymol had weak EA (36.5 %RME2) only at 10^{-8} M, and undetectable EA at the other five concentrations (absolute %RME2 within 30%). Carvacrol exhibited weak EA in 2 concentrations with %RME2 value 49.6% at 10^{-8} M (1.5 μg/L) and 34.2% at 10^{-12} M (0.15 ng/L) (Figure 3.4A). The carvacrol showed higher EA than those of thymol at 10^{-12} and 10^{-11} M (0.15 and 1.5 ng/L) with 39.8% and 53.8% more of RME2%, respectively. There was no significant difference of %RME2 values between thymol and carvacrol groups from 10^{-10} M to 10^{-7} M (0.015 to 15 μg/L). At the concentration of 10^{-8} M, both carvacrol and thymol showed weak EA. When compared to MCF-7 cell proliferation in the E2 group (the positive control), the thymol treatment showed significant decreases at 10^{-12}, 10^{-11}, 10^{-9}, and 10^{-8} M (EA only at 10^{-8} M), while the deceases only were observed at 10^{-9} and 10^{-8} M in carvacrol group in comparison with E2.

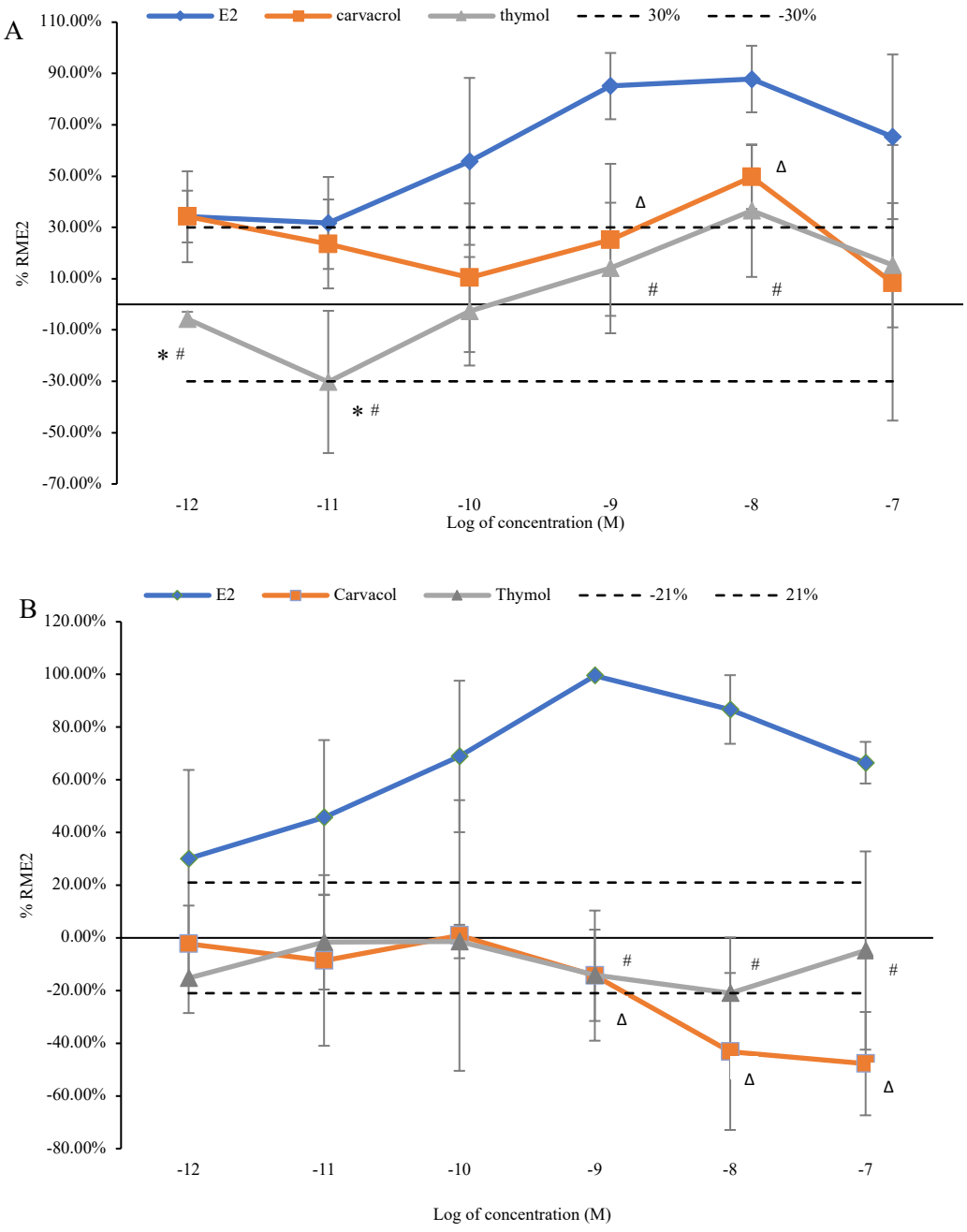


Figure 3.4: The estrogenic activity of E2, thymol, and carvacrol.

Data represented as mean ± standard deviation of at least three independent trials with triplicates in each trial, %RME2 indicates the relative maximum %E2. *means there is a significant difference between thymol and carvacrol; $^{\#}$ means there is a significant difference between thymol and E2; $^{\Delta}$ means there is a significant difference between E2 and carvacrol. A. The EA by MCF-7 cell proliferation assays as conducted by Xinwen Zhang in 2019. B. EA by MCF-7 cell proliferation assays from Ying Peng in 2018. %RME2 value in the control group was set to 0%, and the SD value for Figure 3.4A and 3.4B were 10% and 7%, respectively. Chemicals were tested at 10^{-12} to 10^{-7} M (2.7×10^{-4} to 27 μg/L for E2, 1.5×10^{-4} to 15 μg/L for thymol and carvacrol).

After the results from two researchers in two years were compared, the E2 group has a similar pattern, which showed a maximum increase around 10^{-9} M. However, the findings related to carvacrol and thymol showed some variations. In previous data recorded in 2018 (Figure 3.4B), the %RME2 of carvacrol at 10^{-8} and 10^{-7} M were -43.1% and -47.7%, respectively, while in the recent study recorded in 2019, the %RME2 value of carvacrol at same test concentrations showed 49.6% at 10^{-8} M and 8.4% at 10^{-7} M (Figure 3.4A). Carvacrol had undetectable EA in other 4 test concentrations (Fig 4B) while in recent study carvacrol had weak EA at 10^{-12} M (34.2% for %RME2). Unlike carvacrol, thymol exhibited undetectable EA (absolute %RME2 < 21% in Figure 3.4B) at all six test concentrations in the 2018 study. The weak EA was only detected at 10^{-8} M in the recent 2019 testing. Based on

these findings from the two years of study, the thymol showed a more stable response about EA than those of carvacrol, which showed a more fluctuating pattern.

3.4.3 Thymol and carvacrol showed mutagenic potential in the Ames test

Shown in Table 3.3, the positive control of each bacteria strain, with or without S9, produced a statistically significant increase in the number of revertant colonies, which confirmed the sensitivity and accuracy of the test system. Signs of mutagenicity have been detected for both thymol and carvacrol at some concentrations. After exposure to thymol and carvacrol, no significant increase in the number of revertant was observed for the TA 98 and TA 102 strains at any concentration tested, either in the presence or absence of S9. Significant increases of TA 100 revertant numbers ($p < 0.05$) in the presence of S9 were determined for carvacrol at 10^{-7} M, 10^{-8} M, and 10^{-11} M (15 μg/L, 1.5 μg/L, and 1.5 ng/L) with MI 1.3, 1.5, and 1.3, respectively. Thymol exposure to TA 100 at concentrations of 10^{-6} M (150 μg/L) and 10^{-8} M (1.5 μg/L) significantly increased revertant numbers and had MI at 1.4 and 1.3, respectively. However, no MI exceeded the threshold of 2.0 at any test concentration of thymol or carvacrol, and MI >= 2 is needed for determining genotoxicity (Docherty, Hebbeler, and Kulpa 2006).

Table 3.3: The impacts of thymol and carvacrol 10^{-12} to 10^{-6} M (0.15 ng/L to 150 µg/L) on genotoxicity using the Ames test (0.05 mL of each chemical).

Treatments	number of revertant/ plate in S. typhimurium strains (M ± SD) and (MI)					
	TA 98 (-/+)		TA 100 (-/+)		TA 102 (-/+)	
negative control (0.1% DMSO)	16 ± 4	30 ± 4	72 ± 8	118 ± 4	245 ± 19	302 ± 21
positive control	335 ± 46**	728 ± 100**	733 ± 78**	842 ± 42**	798 ± 49**	997 ± 35**
Thymol						
10^{-6} M (150 µg/L)	22 ± 1 (1.4)	23 ± 5 (0.8)	63 ± 3 (0.9)	164 ± 4** (1.4)	252 ± 11 (1.0)	377 ± 14 (1.3)
10^{-7} M (15 µg/L)	21 ± 2(1.3)	32 ± 5 (1.1)	80 ± 11 (1.1)	128 ± 9 (1.1)	249 ± 3 (1.0)	399 ± 78 (1.1)
10^{-8} M (1.5 µg/L)	19 ± 1 (1.2)	26 ± 3 (0.9)	67 ± 6 (0.9)	154 ± 6* (1.3)	271 ± 7 (1.1)	361 ± 7 (0.9)
10^{-9}M (0.15 µg/L)	13 ± 3 (0.8)	30 ± 5 (1.0)	74 ± 10 (1.0)	95 ± 22 (0.8)	239 ± 6 (1.0)	332 ± 16 (0.9)
10^{-10} M (15 ng/L)	12 ± 4 (0.7)	28 ± 2 (0.9)	68 ± 9 (0.9)	98 ± 7 (0.8)	221 ± 16 (0.9)	313 ± 15 (0.9)
10^{-11} M (1.5 ng/L)	14 ± 4 (0.9)	21 ± 5 (0.7)	68 ± 3 (0.9)	103 ± 15 (0.9)	230 ± 12 (0.9)	296 ± 11 (0.9)
10^{-12} M (0.15 ng/L)	10 ± 1 (0.6)	18 ± 4 (0.6)	70 ± 10 (1.0)	101 ± 5 (0.9)	224 ± 8 (0.9)	295 ± 24 (1.0)
Carvacrol						
10^{-6} M (150 µg/L)	18 ± 4 (1.1)	31 ± 4 (1.0)	72 ± 5 (1.0)	138 ± 8 (1.2)	241 ± 10 (1.0)	339 ± 24 (1.1)
10^{-7} M (15 µg/L)	18 ± 1 (1.1)	34 ± 7 (1.1)	84 ± 8 (1.2)	156 ± 6* (1.3)	248 ± 4 (1.0)	339 ± 7 (1.0)
10^{-8} M (1.5 µg/L)	14 ± 4 (0.8)	29 ± 4 (1.0)	70 ± 5 (1.0)	181 ± 6** (1.5)	223 ± 13 (0.9)	361 ± 9 (1.1)
10^{-9} M (0.15 µg/L)	10 ± 1 (0.6)	24 ± 10 (0.8)	62 ± 5 (0.9)	100 ± 18 (0.8)	224 ± 6 (1.0)	313 ± 15 (0.9)
10^{-10} M (15 ng/L)	12 ± 6 (0.8)	21 ± 4 (0.7)	65 ± 5 (0.9)	98 ± 8 (0.8)	280 ± 2 (1.1)	329 ± 21 (1.1)
10^{-11} M (1.5 ng/L)	11 ± 3 (0.7)	24 ± 4 (0.8)	62 ± 6 (0.9)	159 ± 8* (1.3)	258 ± 7 (1.1)	312 ± 16 (0.9)
10^{-12} M (0.15 ng/L)	11 ± 1 (0.7)	21 ± 6 (0.7)	60 ± 7 (0.8)	123 ± 14 (1.0)	256 ± 10 (1.0)	318 ± 23 (1.0)

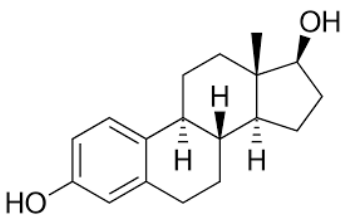
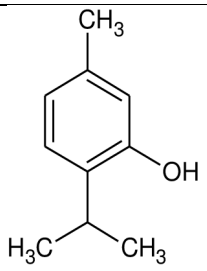
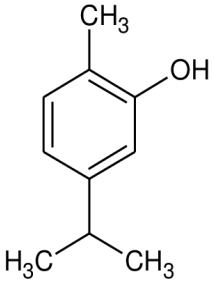
The 0.1% DMSO in PBS was used as a negative control and was the solvent for dissolving test chemicals. Data are shown as mean ± standard deviation (SD)

revertants/plate of two independent trials with two replicates for each concentration in each experiment. Positive control: TA98 without S9: 2-NF (0.1 μg/plate), TA100 without S9: NaN_3 (1 μg/plate), and TA 102 without S9: Mitomycin C (1 μg/plate), 2-AA (5 μg/plate) was used for the strains in the presence of S9. $^{*}p < 0.05$ (ANOVA) significant, and $^{**}p < 0.01$ (ANOVA), M ± SD = mean ± standard deviation.

3.4.4 Docking results of carvacrol showed a relatively higher binding ability to several hormone-related receptors when compared to those of thymol

The binding affinity results showed that E2 had at least a medium binding affinity to agonistic and antagonistic conformations of androgen receptor (AR), ER α, and ER β, along with glucocorticoid receptor (GR), mineralocorticoid receptor (MR), and two thyroid receptors (TR α, TR β) (Table 3.4). On the other hand, thymol and carvacrol showed low binding (green) to most of the receptors except antagonistic androgen receptor (AR an). Compared with thymol, carvacrol had relatively higher bindings to several hormone-related receptors, such as ER α and ER β, MR, and TR α and TR β (Table 3.4). Thymol and carvacrol's binding affinity to ER α and ER β is in good agreement with our EA experimental finding using the MCF-7 proliferation assay. This indicated that the *in-silico* results for EA were confirmed by experimental data using a widely accepted EA test. Except the carvacrol's binding to MR, all other bindings of carvacrol were still inside the low binding class. Carvacrol had medium binding ability to MR while thymol had a low binding affinity.

Table 3.4: Binding energy for E2, thymol, and carvacrol for 14 nuclear receptors.

Ligand	structure	Binding energy with receptors (kcal/mol)
E2		AR : -10.5 | AR an.: -10.1 | ER α: -10.6 ER α an.: -10.7 | ER β: -10.0 | ER β an.: -9.2 GR: -9.5 | GR an.: -8.0 | LXR α: -10.4 LXR β: -10.8 | MR: -8.6 | PPAR α: -8.0 PPAR β: -8.2 | PPAR γ: -8.0 | PR: -2.7 RXR α: -8.8 | TR α: -9.6 | TR β: -9.8
Thymol		AR : -7.1 | AR an.: -6.7 | ER α: -6.1 ER α an.: -5.9 | ER β: -6.1 | ER β an.: -6.0 GR: -6.3 | GR an.: -5.9 | LXR α: -6.8 LXR β: -6.9 | MR: -6.6 | PPAR α: -6.2 PPAR β: -6.4 | PPAR γ: -6.0 | PR: -2.6 RXR α: -6.2 | TR α: -6.9 | TR β: -6.6
Carvacrol		AR : -6.6 | AR an.: -7.0 | ER α: -6.3 ER α an.: -6.1 | ER β: -6.2 | ER β an.: -6.2 GR: -6.5 | GR an.: -6.1 | LXR α: -7.0 LXR β: -7.0 | MR: -7.1 | PPAR α: -6.4 PPAR β: -6.5 | PPAR γ: -6.2 | PR: -2.7 RXR α: -6.4 | TR α: -7.0 | TR β: -6.8

There are four probability binding classes: red (high level of binding), orange (good binding), yellow (medium binding), and green (low binding). The 14 nuclear receptors include androgen receptor (AR), estrogen receptors α (ER α) and β (ER β), glucocorticoid receptor (GR), liver X receptor (LXRα, LXRβ), mineralocorticoid

receptor (MR), progesterone receptor (PR), thyroid receptor (TRα, TRβ), peroxisome proliferator-activated receptors (PPAR α, PPARβ, PPAR γ), Retinoid X receptor (RXR), and 4 antagonist conformations, AR an, ER α an, ER β an, GR an.

3.5 Discussion

Thymol and carvacrol, two phenolic isomers regarded as GRAS compounds in food application, still exhibited toxicity risks with different dose response profiles in our study when using the multi-assay approaches with the chicken embryonic assay, MCF cell proliferation assay, and Ames test. Carvacrol showed more adverse effects in embryonic growth, had a high mortality rate at 45.5%, and a low ratio of embryo weight to egg weight ($p < 0.05$) for 50 mg carvacrol /kg group. In MCF-7 cell proliferation assay, carvacrol showed weak EA at two test concentrations 10^{-8} and 10^{-12} M (1.5 μg/L and 0.15 ng/L), while thymol had weak EA at 10^{-8} M (1.5 μg/L) but there was variance within our two years' study using the cellular assay. The Ames test showed that both carvacrol and thymol had signs of mutagenic activity (mutagenic index lower than critical value 2.0) at a tested concentration ranged from 10^{-11} to 10^{-8} M (1.5 ng/L to 1.5 μg/L). In addition, the molecular docking results revealed that carvacrol demonstrated higher bindings to ER α and β, MR, and TRα and TRβ when compared to those of thymol. All the new data demonstrated that the toxicity risks of thymol and carvacrol cannot be neglected in the low concentrations which are related to the human exposure levels. Moreover, carvacrol had more concerns of embryonic

developmental toxicity, endocrine disruption, and mutagenic activity when compared with the findings of thymol.

Even though thymol and carvacrol are regarded safe when used as flavoring agents, the direct incorporation of these compounds as the natural antimicrobials into the food matrix requires an evaluation of their safety at potential usage and exposure concentrations (Engel et al. 2017). Previous studies on the toxicities of thymol and carvacrol were focused on a relatively high concentration range (above 100 mg/kg or 1 μM). For example, in a recent study on the bioherbicidal potential of thymol and carvacrol, both chemicals exhibited phytotoxic, cytotoxic, and genotoxic activities, including inhibition of plant germination and growth, along with mitotic index reduction and nuclear alternation increase in cells after exposure to thymol and carvacrol at 5 mM (de Assis Alves et al. 2018). Few reports assessed their potential EA and mutagenic effects especially at low exposures such as 10^{-12} to 10^{-7} M (0.15 ng/L to 15 μg/L). Our study is thus important to better understand the safety and toxicity for thymol and carvacrol at low exposure levels. Furthermore, in our study the NMDR was determined for thymol or carvacrol in three assays. In the Ames test, the highest MI level was observed at 10^{-8} M (1.5 μg/L) for carvacrol in TA 100 strain among test concentrations 10^{-12} to 10^{-6} M (0.15 ng/L to 150 μg/L). In the MCF-7 cell proliferation assay, thymol and carvacrol had maximum EA value at 10^{-8} M (1.5 μg/L) among the test levels 10^{-12} to 10^{-7} M (0.15 ng/L to 15 μg/L). Using the chicken embryonic assay, the low dose of carvacrol treatment (50 μg/kg) exhibited

significantly higher chicken embryonic mortality rate, lower values for the ratio of embryo weight to egg weight, and LSI, when compared with the findings from the higher dose carvacrol group (500 μg/kg) ($p < 0.05$). These implicated effects from lower concentrations cannot be obtained by extrapolation from the monotonic dose-response curve. The NMDR has been reported in natural hormones and other EDCs (Vandenberg 2014).

As described in the Introduction, the estimated average human exposure level (181.8 μg/kg) is between the two human equivalent doses calculated from the two final doses of thymol or carvacrol (50 μg/kg and 500 μg/kg) used in the chicken embryonic assay if the average consumption of thymol or carvacrol-containing food is 100 g and the allowed additions in foods are at 100 mg/kg, fully absorbed by an adult with an average body weight of 55 kg. Thymol and carvacrol are often added in candy, ice cream, and beverages, which appeal more to children. It's possible that the total amount of thymol or carvacrol containing food intake is higher than 100 g for some populations and exposure levels to the compounds are higher for people consuming a lot of confectionery or chewing gums or many different kinds of carvacrol or thymol-containing food products daily. According to the Agency for Toxic Substances and Disease Registry (ATSDR), the effective dose in chicken should be multiplied by a factor of 18.5 to derive human equivalent dose (mg/kg/day) (ATSDR. 2011). As suggested by Nair & Jacob (2016), dose extrapolation from animals to humans requires consideration of body surface area, the kinetics of test compounds and

physiological time due to unique characteristics on anatomical, physiological, and biochemical process among species. In order to extrapolate the adverse effect of a toxicant from animals to humans, the same dosage (mg/kg bw) and the body area (mg/m^2) serves as the basic factor, and it is considered humans are 10 times more sensitive than animals. So, the human equivalent dose is then divided by a factor value of 10 to increase human safety. Considering these two factors, the corresponding human equivalent doses (92.5 and 925 μg/kg) can be calculated from the two doses in the chicken embryo (50 and 500 μg/kg) by dose conversion equation (1) in the method section. Moreover, the two doses of 50 μg/kg and 500 μg/kg agreed with the published relevant dose range (approximately 75 to 300 μg/kg) in chicken embryo model for EDC toxicity evaluation (Jessl, Scheider, and Oehlmann 2018). Therefore, the two concentrations (50 and 500 μg/kg) in our chicken embryonic assay were related to the human exposure levels. The exposure level of two GRAS compounds in children would have been larger due to the higher consumption and lower body weight, making our studies on test doses relevant. The embryos exposed to the higher dose of E2, and the lower dose of BPA and carvacrol all resulted in a significant increase in embryonic mortality rate from 12.5% to 50% when compared with that in the solvent control. The mortality rate of lower dose carvacrol group was 45.5% (50 μg/kg), similar to the finding from the E2 group at 50% (91 μg/kg), and higher than the rate in its higher dosage (500 μg/kg) group, indicating the potential NMDR for carvacrol.

In addition to the apparent adverse outcome of increased mortality, other developmental endpoints, including the ratio of embryo weight to egg weight, LSI, liver weight, and the level of lipid peroxidation, were impacted by the lower dose of carvacrol' treatments, again suggesting the potential NMDR for carvacrol. The ratio of embryo weight to egg weight of lower dose of carvacrol group (50 μg/kg) was significantly lower than that of the solvent control group (p <0.05), and the average liver weight (0.43 ± 0.03 g) in 50 μg/kg carvacrol group is 14% lower than control (0.50 ± 0.04 g) although no statistical difference was detected. The initial individual egg weight can influence the final embryo weight, even though we used a similar average egg weight for each group. The correlation between egg weight and embryo weight also varied depending on the embryo development stage (Schmidt et al. 2009). Still carvacrol and thymol demonstrated distinctly different negative impacts on chicken embryos, with more toxicity from carvacrol treatments. The inhibition of ATP synthesis and increased reactive oxygen species (ROS) production are the two possible pathway mechanisms resulting in cell necrosis, which lead to adverse impacts in chicken embryo development by a lower dose of carvacrol but remains to be proved (de Assis Alves et al. 2018). We did detect the non-statistically higher MDA levels in lower doses of carvacrol treatment when compared to its higher dose treatment.

We assessed the MDA level to determine the oxidative damage in chicken embryo livers (G. Chen et al. 2015). MDA level was used to detect cell membrane ROS-mediated damage in rat pancreatic tissue in a previous study, and carvacrol at a dose higher than 100 mg/kg increased the MDA values significantly (Stojanović et al.

2019). In our study, we focused on the much lower concentration of thymol and carvacrol (50 and 500 μg/kg) and compared treatment findings to the findings by the positive controls (E2, BPA) and solvent control for a better understanding of their toxic impacts. Results showed that the solvent control group had the lowest MDA value at 60.37 ± 12.25 nmol/g, and all the treatment groups showed increased numbers from 81.26 to 114.83 nmol/g. The significantly increased MDA values were observed in the higher dose of BPA (76.7 μg/kg) and lower dose of E2 (9.1 μg/kg) treatments ($p < 0.05$). A positive association between BPA exposure and oxidative stress was reported in a previous study (Yang et al., 2009). In the study, urinary BPA concentrations were positively related to urinary MDA and 8-OHdG levels in all three tests of the postmenopausal women. Additionally, exposure to the lower dose (50 μg/kg) of carvacrol lead to increased MDA levels than those of the VC group (Figure 3.3, $p < 0.05$). Interestingly, we observed that the lower dose of E2, thymol, and carvacrol treatments showed higher levels of MDA values compared to their higher dose treatments. For E2, the value was 113.49 ± 28.02 in the lower dose group and 101.80 ± 4.10 nmol/g for the higher dose group. For thymol treatments, the MDA value of 50 μg/kg group was 95.35 ± 24.69 and 81.26 ± 13.42 nmol/g for 500 μg/kg group; while the lower dose (50 μg/kg) of carvacrol treatment had a higher MDA value than that in 500 μg/kg group, with 109.50 ± 16.86 and 82.45 ± 27.33 nmol/g, respectively. Although there was no significant difference between the two doses for each of the chemicals, the changes warrant further investigations to fully understand

the potential NMDR for the test compounds. The similar MDA values between thymol and carvacrol at the same concentration indicated that their different toxic effects on chicken embryos did not attribute to the lipid peroxidation.

Interestingly, within the two doses of carvacrol treatments, the ratio value of embryo weight to egg weight in 50 μg/kg group was significantly lower than that in the higher dose (500 μg/kg) group ($p < 0.05$). Considering the higher mortality rate in a lower dose of carvacrol group, the lower dose of carvacrol induced more adverse effects during chicken development compared with the higher dose treatment. On the other hand, thymol treatment did not show any adverse impacts on this ratio and mortality rate. It is important to notice that the different responses between thymol and carvacrol were determined even though these two chemicals are isomers, just having one different position of a phenolic hydroxyl group. The underlying mechanisms for the isomers to show different toxic effects on chicken embryos remain to be studied, and it is likely unrelated to the lipid peroxidation as there are similar MDA values between thymol and carvacrol at the same concentrations. Furthermore, a previous study showed that no free thymol is detected in human plasma or urine after injection of a thyme extract tablet, and only thymol sulfate was identified in human plasma at a maximum 93.1 ± 24.5 μg/L while the thymol sulfate and thymol glucuronide could be identified in human urine (Kohlert et al. 2002). This finding warrants further investigation on the toxicity of thymol metabolites. On the other hand, the metabolism of carvacrol is not available in humans (Sharifi-Rad et al. 2018).

The MCF-7 cell line is an important and widely applied model for estrogenic activity evaluation at the whole-cell level. The EDCs, such as BPA, can mimic the effects of estradiol on cell proliferation to promote the growth of MCF-7 cells (Martin et al. 2003). In our experiments, the E2 effect in MCF-7 cells from 10^{-12} to 10^{-7} M (0.15 ng/L to 15 μg/L) agreed with the previously reported data (Yang et al., 2014; Peng et al., 2018). The two studies conducted in 2018 and 2019 by two researchers in the same research group also obtained a similar pattern for E2 treatments but showed variations for the thymol and carvacrol tested at the same concentrations. In the 2018's study the %RME2 of carvacrol at 10^{-8} and 10^{-7} M (1.5 and 15 μg/L) were -43% and -48%, while in 2019's study the %RME2 value of carvacrol at the same test concentrations were 50% and 8% respectively. For thymol, even though there were positive and negative fluctuations, only at 10^{-8} M (1.5 μg/L) showed weak EA (with more than 30% of RME2% > 3 SD of the assay VC); others all showed undetectable EA in a recent study which was consistent with results conducted in 2018. This situation is not rare in cell-based *in vitro* bioassays (Hirsch and Schildknecht 2019). A recent study reported that a 200-fold variation was found in growth inhibition rates of the MCF10A cell line, even though they used the same stock of cells and chemicals (Niepel et al. 2019). In our two years' study of thymol and carvacrol using the MCF-7 cell proliferation bioassay, several factors could contribute to the data variations. For example, different stocks and passages of the MCF-7 cells were used in the studies even though both researchers used the newly purchased MCF-7 cells from American

Type Culture Collection (ATCC No. HTB-22). In 2018, Ying used a passage of cells around 10-13 while in 2019, Xinwen used the MCF-cell passages at 3-5 in the MCF-7 cell proliferation bioassay. Besides, even though the same protocol was used by two researchers in our research group, the interpersonal subtle operational differences were difficult to be completely excluded. However, the exact underlying mechanism remains to be studied. We hypothesize that the MCF-7 proliferation assay has inherent limits and variations for some chemicals such as carvacrol and thymol. Our findings confirmed that more work is still needed to standardize the MCF-7 proliferation assay for the EA test. Even though the MCF-7 cell proliferation assay was nominated for several times to assess the EA (Inc CertiChem 2004; C. Z. Yang et al. 2014; Kleensang et al. 2016), it is not approved as a standard method to test EA.

Thymus Caramanicus extract which contained carvacrol (51.0%) and thymol (20.84%), at different concentrations (80, 100, 150, 200, 250, and 300 mg/L) significantly decreased MCF-7 cell viability. The concentration of carvacrol in the study was equal to 0.26 ~ 1 mM (Esmaeili-Mahani, Falahi, and Yaghoobi 2014). The thyme essential oil, with thymol as a major component (10 – 64%) (Salehi et al. 2018), showed strong anti-cancer ability towards human cancer cell lines including PC-3, A549, and MCF-7, with 50% inhibition concentration (IC50) on MCF-7 cell at 0.030% (v/v) (Zu et al. 2010). In a recent study, thymol induced apoptosis in MCF-7 cells at 5 to 100 mg/L with the IC50 values at 54 mg/L (Seresht et al. 2019). The potential anti-cancer properties of thymol or carvacrol might influence its response in the current MCF-7 proliferation assays for EA tests, but the exact impacts remain to be

studied at much lower levels. Thymol and carvacrol ranging from 10^{-12} M to 10^{-7} M (0.15 ng/L to 15 μg/L) were used in the MCF-7 proliferation assay for EA, compared with 39 mg/L to 150 mg/L (0.26 mM to 1 mM) used in the cytotoxicity and anti-cancer assay in the previous studies. In addition, the cell culture condition was different in the previous anti-cancer assays when compared with the one used in our study. In the anti-cancer assays, the regular DMEM and FBS were used in an MTT assay, while in the MCF-7 cell proliferation assay for EA, the phenol red-free DMEM and 5% charcoal stripped FBS were used, which exclude the estrogen, estrogenic compounds, and other hormones interferences from the medium. Even though thymol and carvacrol only differ in one location of one phenolic hydroxy group, they responded quite differently in MCF-7 cells. Carvacrol showed EA at 10^{-12} M (0.15 ng/L), and the %RME2 values of carvacrol were higher than those of thymol. NMDR is again demonstrated in the MCF-7 proliferation assay in addition to the findings in test of the embryonic assay, which implicated effects from lower concentrations should be evaluated separately, not by extrapolation from the monotonic dose-response curve.

It is widely believed that endocrine disruptors' action is through binding to various relevant nuclear receptors. Therefore, we used an *in silico* structure-based method of molecular docking to reveal the potential mechanism of thymol and carvacrol in hormone disruption. The binding affinities of thymol and carvacrol to 14 typical target receptors of EDCs were calculated by software package AutoDock Vina at the DoTS platform. Additionally, E2 was included for comparison since it served as

a positive control in both chicken embryonic assay and MCF-cell proliferation assay. The docking results showed that E2 had a high binding ability to several receptors (AR, ERs, MR, and TRs) as we expected, and the different position of one hydroxy group resulted in different binding affinity to these hormone receptors for thymol and carvacrol. Compared to thymol, carvacrol was easier to bind to two ERs and TRs even though both chemicals were placed inside the low binding class. Carvacrol had a medium binding ability to MR, while thymol showed a low binding affinity. Moreover, these *in silico* docking results could help explain the different responses between thymol and carvacrol in the MCF-7 proliferation assay, in which carvacrol showed higher EA than that of thymol. The phenolic hydroxyl group in the meta position to the methyl group in thymol structure might contribute to the low binding affinity but that remains to be further investigated. As reported, the estrogen receptor-dependent pathway exerts an essential role in the proliferative effect of MCF-7 cells (Okubo et al. 2001; Liao et al. 2014). Our experimental findings of weak or minimal EA of both compounds using the MCF-7 proliferation assay confirmed the thymol and carvacrol's low binding affinity to ERs from the *in silico* results. More experimental validation for other receptors such as AR will be studied in the future.

The effect of thymol and carvacrol on other EDC targeted receptors has been reported in others' studies, such as on the thyroid and androgen receptors. In a rat model, after subcutaneously injecting thymol at 500, 750, 1000 mg/kg doses, the decreased hormone level in T3 and T4 and an increased level of TSH has been observed (Luaibi 2017). The extract of Thymus vulgaris was shown to exert anti-

thyrotropic effects in rats, causing a decline in thyroid-stimulating hormone (Basch et al. 2004). In addition, the anti-androgenic activity was observed for thymol treatment, which inhibited the AR-mediated transcriptional activity induced by 0.125 nM testosterone at 1 and 10 μM (Chen et al. 2007). A recent paper reported that thymol was able to inhibit androgenic signaling pathways in the androgenic yeast assay (IC50 = 73 μM) (Michalíková et al. 2019). In addition, thymol showed antiestrogenic activity at 30 – 1300 μM in the recombinant BMAEREluc/ERα yeast assay, and antiandrogenic activities at 6.0 – 300 μM using the luminescence AIZ-AR test, respectively (Michalíková et al. 2019) . Carvacrol has been proved as an activator of PPARα and γ (Hotta et al. 2010). These findings agree well with the *in-silico* tests on medium binding affinity to androgen receptor antagonist but vary with the low binding affinity to the thyroid receptor.

Potential ED of essential oils including oregano and thyme essential oils were evaluated in a recent study for transcriptional activities of GR, AR, and vitamin D receptor (VDR), which are representatives of the sex hormone-activated steroid receptor, corticoid hormone- activated steroid receptor and nuclear receptor, respectively (Bartoňková and Dvořák 2018). None of these compounds had the toxicological significance at the test concentrations from 0.01 μg/mL to 250 μg/mL. It is notable that different concentrations were used, and mixtures of essential oil compounds were tested not just thymol or carvacrol, when compared with our current study. When using a recombinant yeast assay to determine the estrogenic and antiestrogenic activity of seven natural essential oils, the results showed that the T.

vulgaris essential oils with thymol (52.61%) as the major component, exhibited weak estrogenic activity (higher than 20% of the control) at 0.00001 to 0.1 μL/mL (Contini et al. 2020).

Carvacrol apparently possessed a mutagenic risk at wider and lower concentrations, 10^{-7} M, 10^{-8} M, and 10^{-11} M (5, 1.5, and 0.0015 μg/L), while thymol had mutagenic risks at narrower and higher concentrations 10^{-6} M and 10^{-8} M (150 and 1.5 μg/L). Although some studies showed that thymol and carvacrol had no mutagenicity but exerted antimutagenic activity in *Salmonella* typhimurium strains TA98 and TA97, the evaluations were performed under relatively high concentrations (> 15 μM) (Aicha et al. 2008). In a previous report (LLana-Ruiz-Cabello et al. 2014), no toxic response of thymol in any of the five strains (TA97A, TA98, TA100, TA102, and TA104) was determined at concentrations ranging from 15.6 to 250 μM; while the carvacrol exhibited potential mutagenic activity (MI > 3.8) at concentrations from 29 to 460 μM. Carvacrol was a strong direct-acting mutagen in the bacterial system, with mutagenicity on TA 98 and TA 100 at concentrations from 0.01 to 0.5 μL/plate, MI at 3.7 and 4.5 for TA 98 with or without S9 at 0.01 μL/plate (Ipek et al. 2005). The mutagenic activity of carvacrol, to some extent, was attributed to the production of oxidative DNA damage in purine bases while thymol did not (Llana-Ruiz-Cabello et al. 2014). At the exposure range from 6.25 to 25 μL/plate for thymol and carvacrol, the increased revertant number has been found up to 1.5 - 1.7 times in TA100 and TA98 strains, regardless of metabolic activation (Stammati et al. 1999). However, no previous reports studied the mutagenic effects of thymol and carvacrol at much lower

concentrations from 10^{-12} to 10^{-6} M (0.15 ng/L to 150 μg/L) as tested in our study. Even the highest dose used (1 μM) in our study, approximately equaled to 0.0072 μL/plate, was still lower than the lowest test concentration in other reported studies. Additionally, there were contradictory results on their genotoxicity using different methods, and it was not surprising that all the test concentrations were still above 1 μM (150 μg/L) for thymol and carvacrol. One *in vivo* MN-comet assay using isolated cells from the stomach and liver of rats detected no genotoxicity of carvacrol at the 81–810 mg/kg doses (Llana-Ruiz-Cabello et al. 2016). Another study revealed that no DNA damage was found in the V79 fibroblast cells when treated with carvacrol (1 to 25 μM) and thymol (1 to 5 μM), while increased DNA damage was detected at 25 μM of thymol using the Comet test (Ündeğer et al. 2009). The genotoxic risk has been reported for thymol and carvacrol, which induced the chromosome abnormalities in the bone marrow cells of rats fed at 10 - 100 mg/kg of each compound (Azirak and Rencuzogullari 2008). Based on our study, when a lower concentration range from 10^{-12} to 10^{-6} M (0.15 ng/L to 150 μg/L) was tested by the Ames test, there is no doubt that thymol and carvacrol exert different mutagenic activity even if they are isomers. Further investigations are necessary to explore the mutagenicity mechanisms for these two isomers.

Different findings were determined between thymol and carvacrol by four tests used in our study. In the chicken embryonic assay, thymol had lower mortality rates than those of the carvacrol treatment, while carvacrol showed similar rates with the

finding of E2 treatment. Additionally, thymol and carvacrol showed similar adverse effects in chicken embryonic liver oxidative stress levels with nonstatistically increased MDA values. Both decreased ratio values of embryo weight to egg weight, but only the lower dose of carvacrol had a significant decrease. Carvacrol showed the EA in a wider range of concentrations in MCF-7 human cells when compared to thymol. The estrogenic activity responses agreed with our molecular docking results, in which carvacrol showed higher binding affinity than those of thymol. Finally, in the Ames test, both thymol and carvacrol showed signs of mutagenicity risks (MI > 1.3), at a lower tested concentration range from 10^{-12} M to 10^{-6} M (0.15 ng/L to 150 μg/L). In the future, other bioassays on different target receptors of EDCs, such as androgen receptor and thyroid receptor, should be included to reveal endocrine disrupting activity thoroughly, and to confirm the molecular docking results. The compound metabolites are also an important part of the toxicity profile for a test compound. Furthermore, more research on toxicity mechanisms about thymol and carvacrol, human digestion, and absorption fates along with their metabolite compounds, need to be evaluated in the future for better assessment of their toxicity and ED potential.

3.6 Conclusion

Thymol and carvacrol are regarded as safe when used in food products. However, thymol and carvacrol share a similar structure with 17 beta-estradiol (major female sex hormone), and might be chemicals with potential ED, which have been reported to exert negative health impacts on humans even at very low exposure levels.

In this study, we used the multi-assay approaches, including the chicken embryonic assay, MCF cell proliferation assay, and the Ames test, to assess the toxicity and potential ED of thymol and carvacrol at low concentration range. As shown in the results, these two natural phenolic compounds do show safety warning signs at low concentration ranges. Compared to thymol, carvacrol showed more adverse effects on embryonic growth, had a high mortality rate at 45.5% and a low ratio of embryo weight to egg weight ($p < 0.05$) for 50 μg/kg group. In the MCF-7 cell proliferation assay, carvacrol showed weak EA at 10^{-8} M and 10^{-12} M (1.5 μg/L and 0.15 ng/L), while thymol only had weak EA at 10^{-8} M (1.5 μg/L). The Ames test showed that both carvacrol and thymol only had signs of mutagenic activity (MI< critical value 2.0) from 10^{-12} to 10^{-6} M (0.15 ng/L to 150 μg/L). After the potential endocrine disrupting nature of thymol and carvacrol was systematically assessed on the DoTS platform, both compounds had low binding to ER α and ER β, and carvacrol had medium binding ability to MR. Based on our findings, the low concentration range of thymol and carvacrol showed toxicity, with more toxic effects from carvacrol.

Author Contribution Statement

Xinwen Zhang: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft. Ying Peng: Investigation, Data curation, Writing - review & editing. Changqing Wu: Conceptualization, Methodology, Data curation, Supervision, Writing - review & editing, Project administration.

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Chapter 4

IN SILICO, IN VITRO AND IN VIVO EVALUATION OF THE DEVELOPMENTAL TOXICITY, ESTROGENIC ACTIVITY AND MUTAGENICITY OF FOUR NATURAL PHENOLIC FLAVONOIDS AT LOW EXPOSURE LEVELS

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4.1 Abstract

Thymol and carvacrol are phenolic isomers with potential developmental toxicity and flavonoids are bioactive phenolic compounds widely presented in plant food and used in various nutraceutical, pharmaceutical, and cosmetic products. However, recent studies showed rising concerns of endocrine disruptions and developmental toxicities for many flavonoids. To understand the impacts of flavonoid structure on toxicity, we used a new multitiered platform to investigate the toxicities of four common flavonoids, luteolin, apigenin, quercetin, and genistein, representing flavones, flavonols, and isoflavones. Weak estrogenic activity was detected for the four flavonoids at 10^{-12} to 10^{-7} M by the MCF-7 cell proliferation assay, which agreed with the molecular docking results. Consistent with the Toxicity Estimation Software Tool simulation results, genistein and luteolin showed high developmental toxicity in the chicken embryonic assay (45 - 477 μg/kg) with the mortality rate up to 50%. Luteolin, quercetin, and apigenin showed signs of mutagenicity at 5×10^{-3} pmol/plate. The findings showed non-monotonic dose responses for the chemicals.

KEYWORDS: Flavonoids, Estrogenic Activity, Developmental Toxicity, Mutagenicity, In silico methods, Molecular docking, T.E.S.T. Simulation, MCF-7 Proliferation Assay, Chicken Embryonic Assay, Ames Tests

4.2 Introduction

Flavonoids are a large class of naturally occurring secondary plant metabolites with extensive bioactivities. They are widely found in fruits and vegetables and have been used in nutraceutical, pharmaceutical and cosmetic products due to their health benefits (Patel, Shukla, and Gupta 2007). Among them, luteolin, apigenin, quercetin, and genistein are four typical naturally plant-derived dietary flavonoids. Quercetin can be found in basically all kinds of the berries such as whortleberry (158 mg/kg fresh weight) and chokeberry (89 mg/kg), and an average of 5320 mg apigenin glycosides were found per 100 g dried chamomile flowers (Hostetler, Ralston, and Schwartz 2017a)-(Lakhanpal and Rai 2007). Genistein predominantly presents in soy-based foods, with 5.6 to 276 mg/100 g in mature soybeans (Nabavi et al. 2015). Both aglycone and glycoside forms of flavonoids may exist in foods. The enzymes in human small intestines and the gut microflora can effectively convert flavonoid glycoside to aglycone. The estimated amount of genistein and genistin (its β-glycoside) were 4.6 and 200.6 μg/g beans, respectively. The higher genistein level, 38.5 - 229.1 μg/g food, was detected in fermented soybean products (e.g., miso and natto) (Fukutake et al. 1996). Additionally, genistein glycoside was readily converted to its aglycone form and exerted its biological activities after ingestion (Mizushina et

al. 2013). On average, humans consume approximately 1 g of flavonoids in their daily diet (Formica and Regelson 1995). The estimated intake of these flavonoids via food, commonly fruits and vegetables, is between 0.02 to 3 mg/kg bw/day, but supplementary intake can increase it up to 23 mg/kg bw/day (Manach et al. 2004).

The promising biological activities of natural flavonoids make them receive increased attentions (Tresserra-Rimbau, Lamuela-Raventos, and Moreno 2018)- (Tarragon and Moreno 2020), while the 'natural' term of flavonoids has occasionally misled the consumers' perceptions to overlook their possible adverse effects. Researchers already reported that some of these plant-derived flavonoids exhibited hepatotoxicity, pro-oxidant activity, and potential estrogenic activity (EA) (Galati and O'Brien 2004). Flavonoids could have potential EA since most of them have similar structures to the major female sex hormone-17β-estradiol (E2). Some flavonoids, especially isoflavones, are also called phytoestrogens and may disrupt the normal hormone balance in adolescents or children (Liu et al. 2019). One possible negative health outcome from the disruption of the hormone balance is impairment on reproductive functions, and the antifertility potential has been reported for quercetin or quercetin rich extracts (*Thevetia peruviana*) with a reduced progesterone production in a female Sprague-Dawley rat uterus model (Samanta, Bhattacharya, and Rana 2016). Additionally, the adverse effect of flavonoids on early life stage has been reported using a zebrafish model, with 15 out of 24 flavonoids including apigenin and genistein showing developmental toxicity at 1 - 50 μM (Bugel, Bonventre, and Tanguay 2016). The mutagenicity of quercetin, and the risk of mutagenicity of luteolin and fisetin,

were reported using the Ames test (Flavia Aparecida Resende et al. 2012). Most of the previous findings were related with high exposure levels.

Because of the wide presence in the fruits and vegetables, different flavonoids are important components of our daily diets and many people consider that consumption of these natural flavonoids can benefit human health due to their beneficial bioactivities (Hui et al. 2013). However, the relationships and mechanisms between their chemical structures and potential toxicities are not well studied and only a few studies focused on their potential adverse effects on human health using higher exposure levels. Bioavailable flavonoids are in the ranges of nM to low μM and their plasma concentrations are less than 1 μM (Tresserra-Rimbau, Lamuela-Raventos, and Moreno 2018). To understand how the flavonoid chemical structures and low exposure levels impact their potential toxicities, we selected four common flavonoids in this study, from three subclasses (flavone: apigenin and luteolin; flavonol: quercetin; isoflavone: genistein, Figure 4.1) and investigated their EA, developmental toxicity, and mutagenicity. These flavonoids have low bioavailability making low exposure levels as used in our study highly possible. We included two *in silico* simulations as the first toxicity evaluation to choose the chemicals before the experimental approaches, due to the low-cost and fast speed of the simulations. We assessed the binding affinities of these flavonoids to 14 human nuclear receptors that are the common targets of endocrine disrupting (ED) chemicals (Kolšek et al. 2014). Recently, the chicken embryo has been recognized as a model to bridge the gap between cell-based and animal-based methods and has become an attractive

alternative to in vivo assays under the 3Rs (Replacement, Reduction, and Refinement) guidance (Törnqvist et al. 2014)-(Fonseca, da Silva, and de Morais Ribeiro 2021). We also utilized Toxicity Estimation Software Tool (T.E.S.T.) for prediction of the developmental toxicity and mutagenicity. To confirm the findings of the computational methods, we used the MCF-7 cell proliferation assay for EA, chicken embryonic assay for developmental toxicity, and Ames test for mutagenicity. This study is important as it evaluates the efficacy of our new toxicity method for the common natural compounds with similar structures. After validating the effectiveness of our new approach, we will study other flavonoids more efficiently in the future.

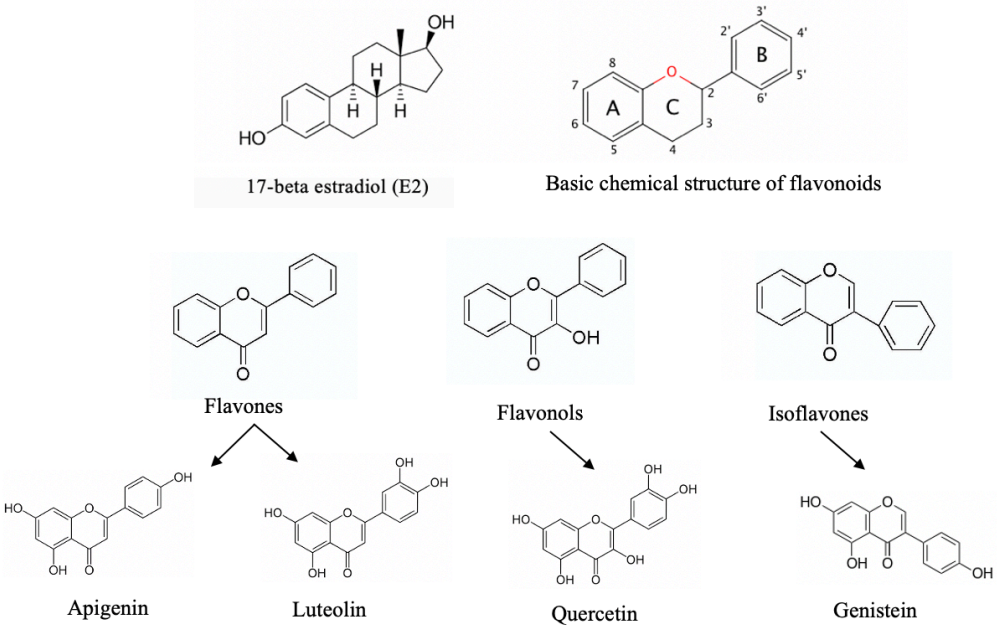


Figure 4.1 Chemical structures of flavonoids

4.3 Experimental Section

4.3.1 Chemicals and cell lines

Luteolin, apigenin, quercetin, genistein, 17β-estradiol (E2), dimethyl sulfoxide (DMSO) (D1391), and phosphate buffered solution (PBS) (Gibco, 20-012-027) were purchased from Fisher Scientific (Waltham, MA). The three *Salmonella* typhimurium tester strains (TA98, TA100 and TA102), top agar, Oxoid Nutrient Broth No.2, S9 mixture solutions were purchased from Molecular Toxicology Inc (Boone, NC, USA). MCF-7 cells were purchased from American Type Culture Collection (ATCC No. HTB-22). Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Cat. No.: 12-430-054), phenol red-free DMEM (Gibco, 21-063-029), fetal bovine serum (FBS) (Gibco, 16-140-071), charcoal stripped FBS (Gibco, 12-676-029), penicillin-streptomycin (Gibco, 15-140-148), dimethyl sulfoxide (DMSO) (D1391) and phosphate buffered solution (PBS) (Gibco, 20-012-027) were purchased from the Fisher Scientific, along with the cell culture 96-Well clear polypropylene microplates (Corning, 05-539-200) and polystyrene T-25 flasks (Corning, 08-772-45).

4.3.2 In silico simulations

4.3.2.1 Molecular docking

The endocrine disrupting potential of four common flavonoids (luteolin, apigenin, quercetin, and genistein) was estimated by the docking Interface for Target Systems (DoTS) platform (named endocrine disruptome tool) via AutoDock Vina

(Kolšek et al. 2014)-(Devillers, Bro, and Millot 2015). The SMILES (Simplified Molecular-Input Line Entry-System) files for each test chemicals (ligands) were used for simulating of binding affinity to 14 nuclear receptors described in our previous study (Zhang, Peng, and Wu 2021) and listed in Table S1.

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

T.E.S.T. has been developed by the United States Environmental Protection Agency (U.S. EPA) to allow users to easily estimate toxicity and physical properties using a variety of QSAR methodologies. The predicted toxicity data presented in this study was generated from the Consensus method which was estimated by an average of the predicted toxicities of five QSAR methods, including Hierarchical, FDA, Single model, Group contribution, Nearest neighbor method. The endpoints included: 96-hour fathead minnow LC50, 48-hour Daphnia magna LC50, Tetrahymena pyriformis IGC 50, Oral rat LD 50, Bioaccumulation factor, Developmental Toxicity, and Mutagenicity.

4.3.3 MCF-7 cell proliferation assay

The MCF-7 cell proliferation assay was performed as previously described (Zhang, Peng, and Wu 2021)-(Peng et al. 2018). The cells were treated with five chemicals (luteolin, apigenin, quercetin, genistein, and E2) at six different concentrations ranging from 10^{-12} M to 10^{-7} M in EA free culture medium. The proliferation rate was quantified by measuring the absorbance of MTT (3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) products at 570 nm using a microplate reader (BioTek Synergy ^{2}). Each test was repeated in three independent trials and in triplicate for each trial. The EA of test chemicals is shown as the relative maximum %E2 (%RME2) calculated from the following equation: $100\times (OD \text{ of test} - OD\ of\ VC)/(MAX\ OD\ of\ E2 - OD\ of\ VC)$. The half maximal effective concentration (EC50, by Prism 8) value and estradiol equivalent factor (EEF) (EC50 of E2 divided by EC50 of the sample) were calculated for each compound.

4.3.4 Chicken embryonic assay

4.3.4.1 Egg treatment

In total 144 fertilized Leghorn eggs were obtained from the University of Delaware research farm. The eggs were weighed and divided into eleven groups: VC, and two dosages of each of five compounds. On day 7, the eggs were candled, and a hole was drilled for injection of each chemical solution or VC at 0.2 mL. The chemical solution concentrations were 0.1 mM and 0.01 mM for E2, 0.5 mM and 0.05 mM for luteolin, apigenin, quercetin, and genistein, respectively. The final doses in egg (average of 60 g per egg) included: 91 μg E2/kg, 9.1 μg E2/kg, 476.5 μg luteolin/kg, 47.7 μg luteolin/kg, 450 μg apigenin/kg, 45 μg apigenin/kg, 500 μg quercetin/kg, 50 μg quercetin/kg, 450 μg genistein/kg, and 45 μg genistein/kg. The eggs were randomly assigned on day 0 to each treatment and control groups, with 16 eggs for each of control and two luteolin groups, 22 eggs for each of two E2 groups, and 10

eggs for each of apigenin, quercetin, and genistein groups. More eggs were used in the luteolin groups to validate the findings. On the injection day (day 7), the four unfertilized eggs were removed from E2 91μg/kg and 2 from each quercetin group (50 and 500 μg/kg), and not recorded, resulting in different total number of embryos in Table 1. The hole was sealed with Duco Cement and eggs were placed back into the egg incubator at 38 ℃ and 60% relative humidity. Each test was repeated at least in two independent trials.

4.3.4.2 General toxicity

The number of dead and deformed embryos were recorded during the tests. The incubation was terminated on day 18 by placing them in the refrigerator overnight. All embryos were dissected and evaluated for deformation, embryo mass, liver mass, heart mass, ratio of embryo to egg weight (RREW), and liver somatic index (LSI). The TBARs level was evaluated using the TBARS assay kit (from Cayman chemical, USA, Item No. 700870) following the manufacturer's instructions. Each test was repeated in two independent trials and in duplicate for each trial.

4.3.5 Ames test

The Ames test using three *Salmonella typhimurium* tester strains (TA98, TA100 and TA102) was conducted by the preincubation method as previously described (Zhang, Peng, and Wu 2021). Briefly, 0.05 ml of test compounds, 0.5 mL of S9 metabolic activation mixture (or 0.5 mL PBS), and 0.1 mL of bacterial culture were

mixed. After 30 min incubation at 37°C, 2 ml top agar was added, and the mixture was poured onto a plate containing minimal agar. The His^+ revertant colonies on plates were counted manually after 48 h of incubation. The chemical concentrations from 10^{-12} to 10^{-6} M at 0.05 mL in each plate yielded the final concentration from 5×10^{-5} to 50 pmol/plate. Each test was repeated in two independent trials and in duplicate for each trial.

4.3.6 Data analysis

The results were analyzed with the statistical software package JMP (JMP PRO 13). In the MCF-7 cell proliferation assay, the cell proliferation rates were analyzed by one-way ANOVA and followed by the Dunnett's test to compare to the E2 group at each concentration (JMP PRO 13). In the chicken embryonic assay, the morphological, developmental endpoints among groups, and TBARs levels were all determined using a one-way analysis of variance (ANOVA) followed by the Tukey's test for multiple comparisons versus the vehicle control (JMP PRO 13). Changes were considered statistically significant if $p < 0.05$ or $p < 0.01$. In the Ames test, the data (revertants/plate) was assessed by means of the ANOVA, followed by Tukey's test. The 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 control (VC). A tested compound was considered mutagenic if a two-fold increase in the number of mutants (MI ≥ 2) was observed in at least one concentration (Flavia Aparecida Resende et al. 2012). Signs of mutagenicity

means the compound that didn't reach the two-fold increase but showed statistical significance ($p < 0.05$) of revertant number as compared to the VC was defined as having a sign of mutagenicity.

4.4 Results

4.4.1 Molecular docking, developmental toxicity, and mutagenicity results of four flavonoids predicated by two in silico simulation tools.

In this study the molecular docking was conducted to 14 potential EDCs' targeting nuclear receptors, including four antagonist conformations (AR an, ERα an, ERβ an, and GR an). E2, the major type of female sex hormone, served as positive control. Compared to the E2, the four flavonoids had similar binding affinities to androgen receptor (AR) and two thyroid receptors (TRs), and only quercetin showed slightly lower binding affinities to the TRs (Table S1). Genistein had the highest binding affinities to ERα and ERβ, and apigenin had the second highest affinities, but both showed lower binding levels when compared to the affinities of E2. Genistein and apigenin had good binding to ERβ an, while having medium binding to ERα an and ERβ. For ERα, genistein had good binding and apigenin had medium binding. Luteolin had medium binding to ERα, ERα an, and ERβ an, and low binding to ERβ; while quercetin showed low binding to these four ER conformations. Moreover, these four flavonoids all had higher binding affinities to GR and MR than the findings of E2, with medium binding for GR while high binding for MR.

When the oral rat LD50 value is between 300 to 2000 mg/kg, the chemical belongs to the class 4 (UNECE 2015), with class 1 representing the most severe toxicity. In our T.E.S.T. study, the E2, apigenin, and genistein belong to class 4, with higher acute toxicity level than quercetin and luteolin (both in class 5). All four chemicals along with E2 were classified as developmental toxicants, and luteolin showed the highest level of developmental toxicity followed by quercetin and genistein (Table S2). For mutagenicity, quercetin and luteolin are reported as mutagenicity positive.

4.4.2 Four flavonoids demonstrated consistent EA results from the MCF-7 cell proliferation assay when compared with the finding of molecular docking to ERs.

The chemicals were considered to have detectable EA levels, with the absolute %RME2 higher than vehicle control (VC) + 3 SD (C. Z. Yang et al. 2014). In our study, the VC + 3 SD was 18%. E2 demonstrated the highest EA at 10^{-10} M and EC50 at 1.0×10^{-11} M. Genistein showed the highest EA level (from 19% to 48%RME2, Figure 4.2) among the test flavonoids except for 10^{-8} M, with the highest EA at 10^{-12} M (48%, no statistical significance). Apigenin had the second non-statistically higher EA value (from 15% to 27%) than the findings of luteolin and quercetin except for 10^{-11} M ($p > 0.05$). Compared with quercetin, luteolin had a higher %RME2 level at four out six test concentrations (10^{-11} to 10^{-8} M) without statistical significance. Quercetin showed significantly less EA values than E2 at three

concentrations (10^{-11}, 10^{-10}, and 10^{-9} M) ($p < 0.05$). Quercetin had no detectable EA (< 18%) at all test concentrations except at 10^{-11} M.

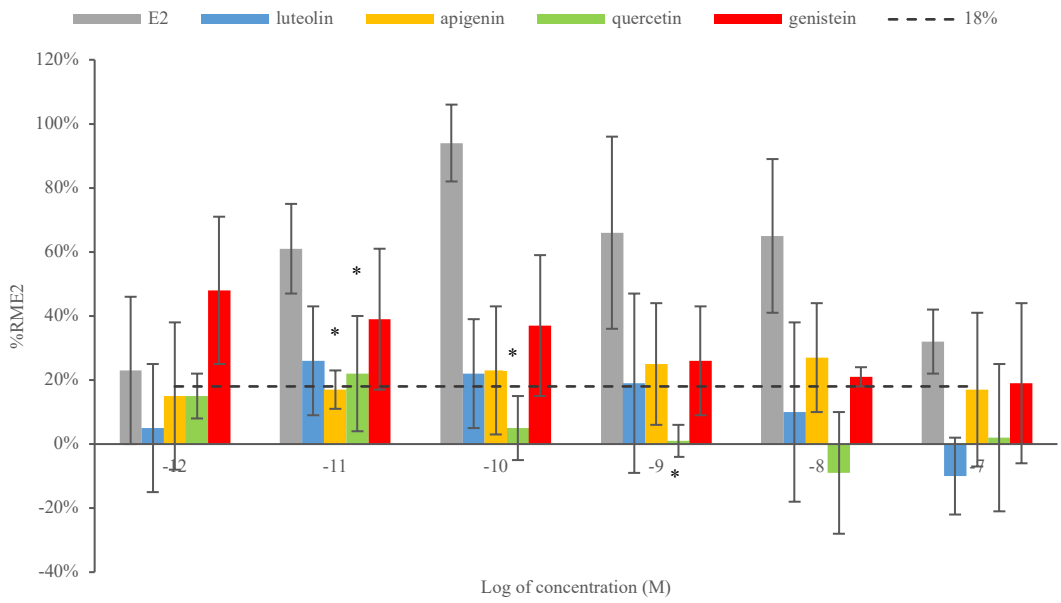


Figure 4.2: The estrogenic activity of E2, luteolin, apigenin, quercetin, and genistein.

Data represented as mean ± standard deviation of at least three independent trials with triplicates in each trial, %RME2 indicates the relative maximum %E2. Differences were evaluated using one-way ANOVA followed by the Tukey's test. *means there is significant difference between test compounds to E2 at same concentration ($p < 0.05$). %RME2 value in the control group was set to 0% and the SD value was 6%. The broadline shown in the figure was VC + 3 SD = 18% (VC: 0.1% DMSO dissolved in cell medium).

4.4.3 Four flavonoids affected chicken embryonic development differently

In this test, 1% DMSO in PBS served as VC and two doses of E2 were used as positive controls. Higher dose (450 μg/kg) of genistein had the highest mortality rate (50%) and was followed by the higher dose (477 μg/kg) of luteolin with 43.8% mortality (Table 4.1). The two doses of E2 (9.1 and 91 μg/kg) and lower dose (45 μg/kg) of genistein had a mortality rate of ~ 30%. Apigenin and quercetin groups showed lower mortality rates (< 25.0%) than the findings for the other two flavonoids, which agreed with the developmental toxicity and rat acute toxicity data from T.E.S.T. The highest malformation rate was found in the 47.7 μg/kg luteolin group at 18.8% with three stunting embryos. The deformed embryo (deformed claw or stunting) was also observed for the high dose of luteolin and quercetin, and low dose of apigenin groups (Figure. S1). The lowest value of REEW (0.33) was detected in the low dose (47.7 μg/kg) of the luteolin group, which was significantly lower than the value of VC group (Table 4.1, $p < 0.05$). Interestingly, the higher dose (450 μg/kg) of genistein had a significantly increased REEW at 0.45 ($p < 0.05$) than the value of the VC group. These results reflect that the exposure to the luteolin impacted mostly the chicken embryonic development; in contrast, the genistein treatment might cause other problems in chicken embryo growth, such as edema. Except for the quercetin 50 μg/kg treatment, the VC group showed the highest LSI value at 2.33%. The LSI values of two apigenin treatments were < 2.00%, with a significant decrease at the lower dosage ($p < 0.05$). Compared with the VC, the higher dose apigenin treatment had a

significantly lower (~ 20%) fetal liver weight ($p < 0.05$). The embryonic heart weight for treatments and controls was similar at approximately 0.19 g.

Table 4.1: Mortality rate, malformation rate, the ratio of embryo to egg weight (REEW), liver somatic index (%), and weight of embryo and organs of chicken embryo on day 18, after injection of E2, luteolin, apigenin, quercetin, and genistein.

Treatments	Injection concentration (mM)	Final dose (μg/kg)	Mortality rate	Malformation rate	REEW	LSI (%)	Weights (g)		
							Embryo	Liver	Heart
VC	1% DMSO	N/A	6.25% (1)	0.0% (0)	0.39 ± 0.063	2.33 ± 0.04	23.18 ± 3.45	0.52 ± 0.03	0.21 ± 0.03
E2	0.01	9.1	31.8% (7)	0.0% (0)	0.39 ± 0.001	2.18 ± 0.01	20.24 ± 3.53	0.54 ± 0.01	0.18 ± 0.03
	0.1	91	33.3% (6)	0.0% (0)	0.38 ± 0.021	2.06 ± 0.38	22.89 ± 0.87	0.47 ± 0.07	0.18 ± 0.01
Luteolin	0.05	47.7	12.5% (2)	21.43% (3)	0.33 ± 0.012*	2.14 ± 0.30	19.75 ± 0.75	0.44 ± 0.05	0.19 ± 0.05
	0.5	477	43.75% (7)	11.11% (1)	0.35 ± 0.017	2.19 ± 0.18	20.70 ± 0.00	0.49 ± 0.05	0.18 ± 0.05
Apigenin	0.05	45	10.0% (1)	11.11% (1)	0.37 ± 0.007	1.81 ± 0.23*	24.29 ± 0.03	0.44 ± 0.02	0.18 ± 0.02
	0.5	450	10.0% (1)	0.0% (0)	0.41 ± 0.021	1.94 ± 0.24	21.49 ± 1.44	0.41 ± 0.02*	0.17 ± 0.02
Quercetin	0.05	50	25.0% (2)	0.0% (0)	0.41 ± 0.035	2.52 ± 0.34	22.11 ± 2.26	0.56 ± 0.13	0.20 ± 0.02
	0.5	500	12.5% (1)	25.0% (1)	0.43 ± 0.057	1.85 ± 0.21	24.06 ± 2.35	0.44 ± 0.01	0.21 ± 0.05
Genistein	0.05	45	40.0% (4)	0.0% (0)	0.41 ± 0.049	2.05 ± 0.44	21.96 ± 0.73	0.46 ± 0.10	0.17 ± 0.01
	0.5	450	50.0% (5)	0.0% (0)	0.45 ± 0.085*	2.12 ± 0.14	23.88 ± 2.02	0.50 ± 0.09	0.20 ± 0.03

The chemical solution (0.1 mM and 0.01mM for E2, 0.5 mM and 0.05 mM for four flavonoids) was injected at 0.2 mL into the egg (average weight 60 g) yielding a

final dose in egg: 91 μg E2/kg, 9.1 μg E2/kg, 47.7 μg luteolin/kg, 477 μg luteolin/kg, 45 μg apigenin/kg, 450 μg apigenin/kg, 50 μg quercetin/kg, 500 μg quercetin/kg, 45 μg genistein/kg, and 450 μg genistein/kg (VC: 1% DMSO dissolved in PBS). The number in parentheses represents the number of dead chicken embryo or malformation chicken embryo. All values are expressed as mean ± standard deviation (SD) from two independent trials. Differences were evaluated using ANOVA followed by the Turkey's test and statistical significance was indicated by $p < 0.05$ (* $p < 0.05$). * means statistically significant difference compared to VC.

The Thiobarbituric acid reactive substances (TBARs) level reflects lipid peroxidation in chicken fetal liver. As shown in Figure 4.3, the VC group had the second lowest value of TBARs at 67.87 nmol/g and the higher dose of the quercetin group had the lowest value at 62.48 nmol/g. A significantly increased TBARs value was observed in two doses of apigenin (45 and 450 μg/kg) groups and the lower dose of luteolin (47.7 μg/kg) group than the values of VC, with values of 103.73, 112.31, and 142.56 nmol/g, respectively ($p < 0.01$). Within the two doses, a significantly higher TBARs level was detected in the luteolin treatment at the lower dose ($p < 0.05$). Generally, the quercetin and genistein groups had lower TBARs levels than the findings for apigenin and luteolin.

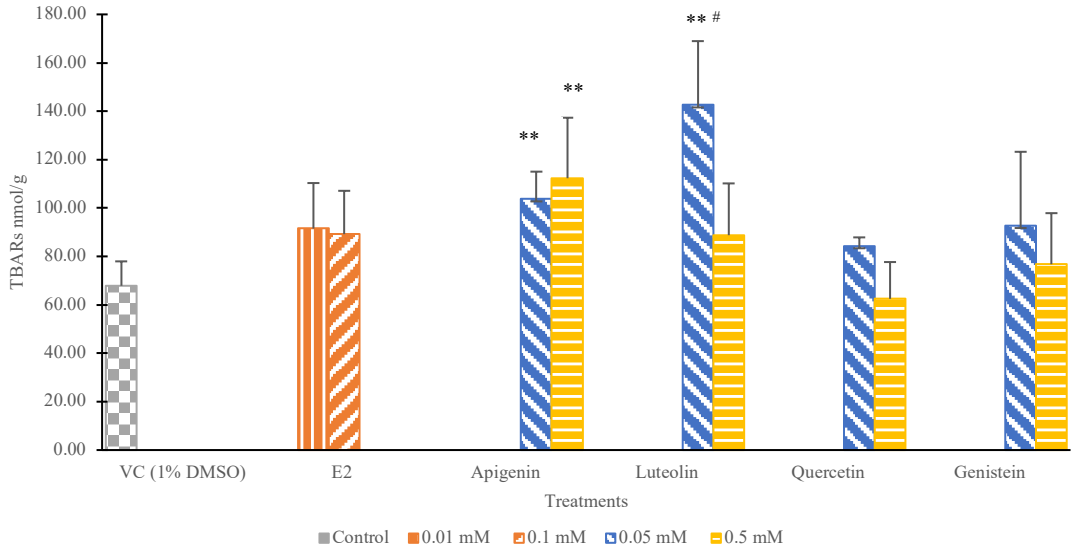


Figure 4.3: Impacts of E2, luteolin, apigenin, quercetin, and genistein on indications of TBARs value for each treatment group (each compound included two inject concentrations).

Values are expressed as mean ± SD from two independent trials performed in triplicate (N=6). The 0.1 mM and 0.01 mM for E2, while 0.5 mM and 0.05 mM for luteolin, apigenin, quercetin, and genistein resulted in the following final concentrations in egg (average of 60 g per egg, each injection at 0.2 mL): 91 µg E2/kg, 9.1 µg E2/kg, 47.7 µg luteolin/kg, 477 µg luteolin/kg, 45 µg apigenin/kg, 450 µg apigenin/kg, 50 µg quercetin/kg, 500 µg quercetin/kg, 45 µg genistein/kg, and 450 µg genistein/kg. Differences were evaluated using one-way ANOVA and followed by the Tukey's test, and statistical significance was indicated by $p < 0.05$ or $p < 0.01$. ** means statistically significant difference at $p < 0.01$ compared to VC (1% DMSO

dissolved in PBS) and # means difference between two doses within one treatment $p < 0.05$.

4.4.4 Different mutagenic index revealed for four flavonoids using the Ames test.

Shown in Table 4.2, the positive control of each bacterial strain with or without S9, produced statistically significant increase in the number of revertant colonies while the VC of each test compound (0.1% DMSO in PBS) had minimal number of colonies, which confirmed the sensitivity and accuracy of the test system. Because these four compounds were conducted in two separated periods, two different sets of negative and positive controls were included for comparison. Significant increase in the number of revertants was observed for all three strains (TA98, TA100, and TA102) after exposure to luteolin. For the TA98 stain, without S9 mixture, luteolin increased the number of revertants at 50, 5, and 0.5 pmol/plate with mutagenic indexes (MI) at 1.9, 1.9, and 1.8, which were very close to the critical mutation value of 2.0. The significant increase of revertants was also observed in TA100 and TA102 strains after exposure to luteolin at 0.05 to 50 pmol/plate with MI up to 1.4. After exposure to the apigenin, a significant increase in the revertants was only observed at TA100 (+S9) and TA102 strains (−S9) at 0.5 to 50 pmol/plate, with the MI up to 1.4. Exposure to quercetin led to a significantly increased number of revertants of TA100 and TA102 strains at four concentrations (5×10^{-3}, 0.5, 5, and 50 pmol/plate) ($p < 0.05$) when compared with VC, with MI up to 1.4. Interestingly genistein didn't

significantly increase numbers for any strains at any test concentration. Comparing with the T.E.S.T. data, the only discrepancy was determined for apigenin. It was classified as mutagenicity negative in T.E.S.T. but showed signs of mutagenic activity in the Ames test.

Table 4.2: Impacts of luteolin, apigenin, quercetin, and genistein on genotoxicity using the Ames test.

Treatments	Number of revertants/ plate in *S. typhimurium* strains (M ± SD) and (MI)					
	TA98 (-/+)		TA100 (-/+)		TA102 (-/+)	
Luteolin (pmol/plate)						
0[a]	10 ± 1	74 ± 10	93 ± 6	306 ± 7	110±11	302±21
50	19 ± 4** (1.9)	88 ± 14 (1.2)	83 ± 6 (0.9)	427 ± 7** (1.4)	150 ± 7** (1.4)	409 ± 7** (1.4)
5	19 ± 2** (1.9)	80 ± 3 (1.1)	97 ± 4 (1.0)	371 ± 21 (1.2)	135 ± 9 (1.2)	422 ± 13** (1.4)
0.5	18 ± 4** (1.8)	85 ± 6 (1.1)	95 ± 6 (1.0)	387 ± 15* (1.3)	144 ± 8* (1.3)	394 ± 10* (1.3)
5×10^{-2}	13 ± 5 (1.3)	75 ± 8 (1.0)	90 ± 4 (1.0)	357 ± 15 (1.2)	126 ± 8 (1.1)	388 ± 14* (1.3)
5×10^{-3}	12 ± 1 (1.2)	79 ± 1 (1.1)	76 ± 13 (0.8)	338 ± 8 (1.1)	129 ± 13 (1.2)	377 ± 15 (1.2)
5×10^{-4}	11 ± 5 (1.1)	75 ± 13 (1.0)	86 ± 11 (0.9)	349 ± 6 (1.1)	126 ± 6 (1.1)	372 ± 37 (1.2)
5×10^{-5}	7 ± 1 (0.7)	64 ± 4 (0.9)	86 ± 4 (0.9)	371 ± 8 (1.2)	113 ± 14 (1.0)	361 ± 25 (1.2)
Positive control	723 ± 64[b]**	846 ± 47[d]**	860 ± 52[c]**	941 ± 56[d]**	854 ± 32[e]**	997 ± 35[d]**
Apigenin (pmol/plate)						
0[a]	10 ± 1	74 ± 10	93 ± 6	306 ± 7	110±11	302±21
50	11 ± 4 (1.1)	75 ± 20 (1.0)	96 ± 8 (1.0)	400±2* (1.3)	147±8* (1.3)	360±13 (1.1)
5	13 ± 1 (1.3)	80 ± 10 (1.1)	96 ± 4 (1.0)	390 ± 4* (1.3)	154 ± 11** (1.4)	364 ± 4 (1.1)
0.5	9 ± 1 (0.9)	85 ± 6 (1.1)	94 ± 4 (1.0)	415 ± 4** (1.4)	142 ± 15 (1.3)	308 ± 19 (1.0)
5×10^{-2}	10 ± 4 (1.0)	87 ± 6 (1.2)	85 ± 6 (0.9)	377 ± 15 (1.2)	131 ± 11 (1.2)	303 ± 19 (1.0)
5×10^{-3}	10 ± 3 (1.0)	80 ± 18 (1.1)	75 ± 13 (0.8)	349 ± 10 (1.1)	124 ± 12 (1.1)	290 ± 20 (0.9)
5×10^{-4}	7 ± 3 (0.7)	83 ± 9 (1.1)	83 ± 6 (0.9)	342 ± 14 (1.1)	114 ± 13 (1.0)	288 ± 14 (0.9)
5×10^{-5}	10 ± 3 (1.0)	68 ± 2 (0.9)	87 ± 6 (0.9)	358 ± 6 (1.2)	126 ± 8 (1.1)	316 ± 19 (1.0)
Positive control	723 ± 64[b]**	846 ± 47[d]**	860 ± 52[c]**	941 ± 56[d]**	854 ± 32[e]**	997 ± 35[d]**

Quercetin (pmol/plate)						
0[a]	22 ± 5	23 ± 13	101 ± 3	322 ± 14	264 ± 28	306 ± 8
50	30 ± 2 (1.3)	18 ± 13 (0.8)	88 ± 8 (0.9)	369 ± 12 (1.1)	347 ± 10* (1.4)	310 ± 31 (1.0)
5	27 ± 11 (1.2)	19 ± 10 (0.8)	104 ± 8 (1.0)	386 ± 12* (1.2)	312 ± 13 (1.2)	279 ± 21 (0.9)
0.5	28 ± 1 (1.3)	25 ± 13 (1.1)	105 ± 11 (1.0)	398 ± 8* (1.2)	267 ± 23 (1.0)	326 ± 19 (1.1)
5×10^{-2}	19 ± 6 (0.8)	23 ± 17 (1.0)	113 ± 6 (1.1)	366 ± 14 (1.1)	202 ± 14 (1.2)	337 ± 7 (1.1)
5×10^{-3}	31 ± 6 (1.4)	27 ± 16 (1.2)	103 ± 7 (1.0)	367 ± 5 (1.1)	325 ± 10* (1.3)	369 ± 13* (1.2)
5×10^{-4}	27 ± 3 (1.2)	18 ± 13 (0.8)	98 ± 7 (1.0)	349 ± 8 (1.1)	301 ± 16 (1.2)	312 ± 16 (1.0)
5×10^{-5}	25 ± 2 (1.1)	17 ± 8 (0.7)	97 ± 4 (1.0)	358 ± 14 (1.1)	284 ± 15 (1.1)	291 ± 11 (0.9)
Positive control	634 ± 16[b**]	806 ± 25[d**]	743 ± 28[c**]	845 ± 45[d**]	740 ± 23[e**]	757 ± 35[d**]
Genistein (pmol/plate)						
0[a]	22 ± 5	23 ± 13	101 ± 3	322 ± 14	264 ± 28	306 ± 8
50	13 ± 4 (0.6)	28 ± 16 (1.2)	80 ± 12 (0.8)	296 ± 11 (0.9)	215 ± 27 (0.8)	324 ± 28 (1.1)
5	18 ± 6 (0.8)	23 ± 14 (1.0)	96 ± 4 (0.9)	301 ± 13 (0.9)	240 ± 8 (0.9)	299 ± 4 (1.0)
0.5	15 ± 1 (0.7)	26 ± 13 (1.1)	103 ± 10 (1.0)	307 ± 7 (1.0)	247 ± 18 (1.0)	336 ± 17 (1.1)
5×10^{-2}	16 ± 2 (0.7)	28 ± 5 (1.2)	97 ± 11 (0.9)	308 ± 8 (1.0)	222 ± 30 (0.9)	311 ± 18 (1.0)
5×10^{-3}	11 ± 1 (0.5)	28 ± 10 (1.2)	100 ± 11 (1.0)	313 ± 11 (1.0)	198 ± 8 (0.8)	312 ± 13 (1.0)
5×10^{-4}	13 ± 6 (0.6)	24 ± 8 (1.0)	94 ± 8 (0.9)	301 ± 4 (0.9)	196 ± 20 (0.8)	285 ± 10 (0.9)
5×10^{-5}	15 ± 4 (0.7)	19 ± 6 (0.8)	98 ± 6 (1.0)	294 ± 6 (0.9)	207 ± 5 (0.8)	290 ± 4 (0.9)
Positive control	634 ± 16[b**]	806 ± 25[d**]	743 ± 28[c**]	845 ± 45[d**]	740 ± 23[e**]	757 ± 35[d**]

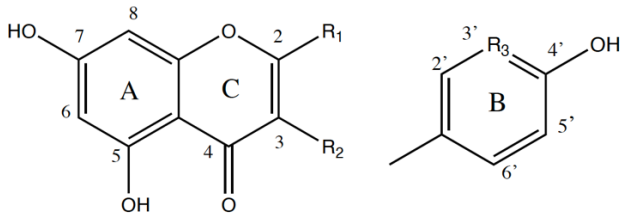
Test concentrations of 10^{-12} to 10^{-6} M at 0.05 mL yield final dosages from 5×10^{-5} to 50 pmol/plate. The 0.1% DMSO in PBS was used as a negative control and was the solvent for the test chemicals (VC). Differences were evaluated using one-way ANOVA followed by the Tukey's test and statistical significance was indicated by $p < 0.05$ and $p < 0.01$ when compared to the negative control, VC. Data are shown as mean ± SD revertants/plate from two independent trials performed in triplicate (N=6). [a]: VC (0.1% DMSO in PBS); Positive controls: [b] 2-NF (1 μg/plate), [c] NaN3 (1 μg/plat), [d] 2-AA (5 μg/plate), and [e] Mitomycin C (1 μg/plate).

4.4.5 The structure-activity relationships (SARs) of four flavonoids

The four flavonoids possess a similar structure backbone with different phenolic hydroxyl groups and different locations of the hydroxybenzene ring B (Table 3 and Figure 1). Compared to the structure of the other three flavonoids, genistein has the hydroxybenzene ring B at position R2 rather than R1, as the characteristic structure for isoflavones. Apigenin and luteolin belong to the flavone subgroup, and luteolin has one more hydroxyl group at the R3 position in ring B. In this study, the EA results showed that genistein had the highest %RME2 value compared to the other three flavonoids at five test concentrations. From 10^{-12} M to 10^{-7} M, the highest %RME2 value of luteolin and quercetin was observed at 10^{-11} M (10 pM); while the values of apigenin and genistein were reported at 10^{-8} M (10 nM) and 10^{-12} M (1 pM), respectively. The ranking of Max %RME2 values were consistent with findings of the docking results to two ERs, with genistein showing the highest Max %RME2 and followed by apigenin and luteolin, while apigenin and luteolin had similar values. The high EA for genistein indicates significant impacts from the hydroxybenzene ring B at position R2. When comparing the EC50 and EEF values, apigenin had the highest values followed by genistein. The EC50 value of apigenin, genistein, and luteolin was 6.2×10^{-11}, 1.2×10^{-10}, and 8.4×10^{-9} M, respectively (Table 4.3). Apigenin and genistein had the top two highest EEF values at 0.16 and 0.08, respectively. The EC50 value was not available for quercetin, since it only had EA at one test concentration, 10^{-11} M. Besides the potential effects of the B ring's location, the numbers of hydroxyl substitutions also play a role in EA potency (from EC50 and EEF values) following

the order: apigenin (three hydroxyl groups) > genistein (three hydroxyl groups) > luteolin (four hydroxyl groups) > quercetin (five hydroxyl groups).

Table 4.3: Estrogenic, developmental, and mutagenic SARs of four flavonoids



Flavonoids			Luteolin	Apigenin	Quercetin	Genistein
Structure	Subgroup		flavone	flavone	flavonol	isoflavone
		R1	Ring B	Ring B	Ring B	H
		R2	H	H	OH	Ring B
		R3	OH	H	OH	H
Experimental tests	MCF-7 cell proliferation	Max %RME2[a]	26%	27%	22%	48%
		EC50 (M)[b]	8.4×10^{-9}	6.2×10^{-11}	NA	1.2×10^{-10}
		EEF[c]	0.0012	0.16	NA	0.08
	Chicken embryonic assay	Maximum Mortality rate[d]	43.8%	10.0%	25.0%	50.0%
		Maximum TBARs value[e] (nmol/g)	142.56	112.31	84.37	92.73
	Ames test	Max MI value[f]	1.9	1.4	1.4	1.2
In silico simulation	Docking to ERs	ER α	-8.6	-8.8	-8.2	-9.2
		Binding class	medium	medium	low	good
		ER β	-7.6	-8.2	-7.1	-8.7

	Binding class	low	medium	low	medium
T.E.S.T results	Oral rat LD50 (mg/kg)	2175.63	1707.99	2782.81	1172.86
	Development value	0.88	0.65	0.77	0.76
	Mutagenicity value	0.53	0.29	0.55	0.23

a: the highest %RME2 from test range at 10-12 to 10-7 M measured by MCF-7 cell proliferation assay

b: EC50 of test compounds was calculated using GraphPad Prism. “NA” means unavailable data, since detectable EA of quercetin only observed in one test dose.

c: EEF was calculated as the EC50 of E2 divided by that of the test compounds. “NA” means unavailable data, since detectable EA of quercetin was only observed at one test dose.

d: the highest mortality rate detected in chicken embryonic assay (detected at higher injection concentration (0.5 mM) for luteolin, apigenin, and genistein; at lower injection concentration (0.05 mM) for quercetin).

e: the highest TBARs value detected in chicken embryonic assay (detected at lower injection concentration (0.05 mM) for luteolin, quercetin, and genistein; at higher injection concentration (0.5 mM) for apigenin).

f: the highest MI value from test range 5×10-5 to 50 pmol/plate measured by Ames test.

The presence of the hydroxyl group at 3' position in B ring (e.g., luteolin) increased the developmental toxicity in the chicken embryo model and the MI value in the Ames test, compared to findings for apigenin, which were also consistent with those of the T.E.S.T. results. Quercetin belongs to the flavonol group with an extra hydroxyl group at position R2. This 2-hydroxyl substitution in the C ring decreased the developmental toxicity in the chicken embryo model and T.E.S.T. simulation. Compared to apigenin, quercetin has two additional hydroxyl groups at 3-position in C ring and 3'-position in B ring (R2 and R3), resulting in a comparable developmental toxicity but higher mutagenic activity in T.E.S.T. (Table 4.3). Genistein has a B ring at 3-position in C ring (R2), which increased the developmental toxicity and decreased MI level, compared to the findings for apigenin.

4.5 Discussion

4.5.1 The binding affinities to EDCs' targeting receptors and EA results from MCF-7 cell proliferation assay indicated endocrine disruption potentials of the test flavonoids.

In this study, we applied for the first time a multitiered platform consisting of *in silico, in vitro,* and *in vivo* tests and compared the *in vitro* or *in vivo* results to the *in silico* data on three toxicity endpoints (EA, developmental toxicity, and mutagenicity) for understanding the impacts of flavonoid structure on toxicities. Few studies have investigated the potential toxicity of flavonoids systematically, especially at the low exposure range. Our study is the first to investigate EA, mutagenicity, and developmental toxicity at low and human exposure related concentration for different

flavonoids subgroups. Our results demonstrated that the EA effect, the sign of mutagenicity, and chicken developmental toxicity were detected at a concentration < 1 μM for different flavonoids. Flavonoids have high potentials to disrupt hormone pathways in the endocrine system, even at low exposure levels and these are not well studied. Considering the binding to the different targeted receptors and the further alteration of the transcription levels as a crucial underlying mechanism of ED, we firstly used the molecular docking of four flavonoids to 14 nuclear receptors to reveal the potential ED of these flavonoids. The ARs, ERs, and TRs with vital roles in the development, growth, and function of reproductive and nonreproductive tissues, were the major targets for EDCs. Our results indicated that the four test flavonoids showed high binding affinities to the two conformational AR structures, which was similar to the findings for E2. Compared with the finding for E2, four test flavonoids showed higher binding affinity levels to MR and similar levels to the GR, which are members of the steroid receptor subfamily and regarded as potential targets for EDCs (Gomez-Sanchez and Gomez-Sanchez 2011). Both are the receptors to adrenal cortical steroid hormones (e.g., aldosterone) and play essential roles in the immune, metabolic, endocrine, and nervous systems. Therefore, in addition to AR, ERs, and TRs, it is highly possible for the flavonoids to exert endocrine disruption effects through other steroid receptors.

Because of the similar structure with 17β-estradiol, some flavonoids (e.g., genistein) are named phytoestrogens, which can bind to ERα and ERβ and exert estrogenic or/and anti-estrogenic effects in mammals. Genistein was reported to

compete with E2 for the ERs with a higher binding affinity for ERβ (87%) and lower binding affinity for ERα (4%) (Banerjee et al. 2008). Apigenin is also a weak phytoestrogen with binding affinities of 0.3% and 6% for ERα and ERβ, respectively (Kuiper et al. 1998). Genistein can induce proliferative activity in MCF-7 cells at 10^{-7} to 10^{-5} M with a maximum value at 10^{-6} M (Murata et al. 2004). Apigenin also demonstrated a strong activating ability for ERα or ERβ than luteolin in ERα or ERβ SK-NBE derived cells, while luteolin only had a slight activating effect on ERβ (Innocenti et al. 2007). The EA of quercetin in previous MCF-7 proliferation assay were contradictory. Quercetin dramatically inhibited MCF-7 cell growth at concentration> 2.5 μM while the stimulation effect was not detected at low concentration ranges (0.5-2.5 μM) (Miodini et al. 1999). In another study (Van Der Woude et al. 2005) quercetin only slightly increased cell proliferation in the MCF-7 cells (< 120%) at 0.001 to 1μM when tested from 0.001 to 50 μM. In our study, quercetin had weak EA of 22% only at the concentration of 10^{-11} M. Most of these previous findings were obtained at higher concentrations than the levels used in our work, yet the highest %RME2 of 48% at 10^{-12} M was still detected for genistein. In addition, it had EA at a wider range from 10^{-12} to 10^{-7} M, compared with findings for apigenin, luteolin, and quercetin.

It is noteworthy that several flavonoids such as genistein and apigenin were reported to have biphasic effects on the proliferation of estrogen-dependent MCF-7 cells in a concentration-dependent manner (Lucki and Sewer 2011) (Allred et al. 2001). High concentrations of genistein (>10 μM) were associated with tumor

suppression, whereas the low concentration range (0.01–1 μM) had a proliferation effect in estrogen receptor positive cells (Hsieh et al. 1998) (Allred et al. 2001). Apigenin is able to stimulate ER-positive breast cancer cell lines (e.g., MCF-7 and T47D cells) with less potent activities than that of genistein from 10 nM to 10 μM (Seo et al. 2006). Similarly, luteolin also acts as a partial agonist that stimulates MCF-7 cell's proliferation at 1 nM to 10 μM (Flávia A Resende et al. 2013).

In our study, the four flavonoids all showed weak EA (max %RME2 from 22% to 48%; Figure 2, Table 3) in MCF-7 cells from 10^{-12} to 10^{-7} M. The highest EA results from the MCF–7 cell proliferation assay were consistent with the molecular docking results to two ERs, following the order genistein > apigenin > luteolin > quercetin, which confirmed that binding to the ERs was one critical pathway in EA. Interestingly, genistein had bigger max %RME2, 21% more than the value of apigenin, but it had half values of EEF or EC50 of apigenin (Table 3). Even though apigenin and luteolin had similar max %RME2 values, EEF or EC50 values for both chemicals varied by 100 times. This indicated EEF or EC50 used in previous publication for ranking the EA effects might not be sufficient as they did not consider the different patterns of response curves (Isidori et al. 2010) (Omoruyi and Pohjanvirta 2018). It is noteworthy that genistein had different dose-response curve pattern in the calculation of EC50 and EEF, when compared with the response curves for apigenin and luteolin using the MCF-7 cell proliferation from 10^{-12} to 10^{-7} M. The highest EA (48%) of genistein was determined at the lowest concentration, while apigenin showed the highest effect (27%) at 10^{-8} M. For luteolin and quercetin, the highest EA values

existed in 10^{-11} M at 26% and 22%, respectively. The maximum EA of E2 (94%) was obtained at 10^{-10} M. The non-monotonic dose-response (NMDR) effect, which has been widely proved in endocrine-disrupting chemicals, was detected for E2, apigenin, luteolin and quercetin, with the highest EA detected at the middle concentrations.

4.5.2 Four flavonoids showed developmental toxicity by in silico simulation T.E.S.T. and chicken embryonic assay.

The *in silico* simulation T.E.S.T. results showed that E2, apigenin, and genistein had stronger acute toxicity (class 4) than luteolin and quercetin (class 5, less toxicity) (UNECE 2015). Furthermore, E2 and the four test flavonoids were classified as developmental toxicants with the order: E2 > luteolin > quercetin > genistein > apigenin. To validate the *in silico* simulation in the T.E.S.T., we assessed the developmental toxicity of four flavonoids using comparable dosages with human daily exposure levels in a chicken embryo model. To derive human equivalent dose (mg/kg/day) from chicken, a conversion factor of 18.5 and a safety factor were used based on the recommendation by the Agency for Toxic Substances and Disease Registry (ATSDR) (ATSDR. 2011) (Nair and Jacob 2016). Thus, the dosages (45 to 500 μg/kg) used in this study can be converted to 83.25 to 925 μg/kg (*human equivalent dose* = (*chemical dose in chicken embryo* × 18.5) / 10), which agreed well with the published human estimated daily intake level of flavonoids at 20 – 3000 μg/kg (Manach et al. 2004). Genistein at 45 and 450 μg/kg, along with luteolin at 477 μg/kg, exerted detrimental effects on chicken embryo development, with a mortality

rate higher than 40%. Low dose luteolin treatment (47.7 μg/kg) induced a 12.5% death rate but a relatively high malformation rate at 18.8% of stunting. On the other hand, the mortality rates and deformation rates were lower after exposure to apigenin and quercetin. Furthermore, other developmental indexes, including the REEW, LSI, and organs weight, were impacted by luteolin, apigenin, and genistein treatments. Our findings on the flavonoids' developmental toxicity confirmed the previous reported results on zebrafish embryo-larval developmental toxicity of apigenin and genistein (Bugel, Bonventre, and Tanguay 2016). Consistent with T.E.S.T. results on classification of developmental toxicants for the four flavonoids, all of them showed adverse effects on the chicken embryogenesis. Luteolin which had the highest developmental toxicity in T.E.S.T., showed a higher mortality rate than apigenin and quercetin.

Significantly increased TBARs values were detected after exposure to the apigenin and luteolin ($p < 0.05$) in our study. As the primary final product of lipid peroxidation, the TBARs level is commonly used as a biomarker to evaluate oxidative damage and has been reported as an important contributor to DNA damage and mutation (Niedernhofer et al. 2003). Our results agreed with the previous finding that apigenin at 100 and 200 mg/kg led to hepatotoxicity in the Swiss mice model, even our dosages were 1000 times lower. The mouse toxicity included increased levels of MDA and ROS, along with altered gene expression levels related to the oxidative stress and apoptosis (Singh et al. 2012). High TBARs values in the apigenin treatment

might be one mechanism to impact the developmental toxicity related to the LSI and liver weight.

Luteolin and genistein showed a high and similar death rate (43.75% and 50%) on chicken embryos especially at the higher dosage groups while the TBARs levels in these two groups were not statistically different ($p > 0.05$). The oxidative stress might not be the only pathway to induce the toxicity. In previous studies, luteolin (at 10 and 20 μM) and genistein had anti-angiogenic activity by inhibiting the Gas6/Axl signaling pathway or targeting at angiostatin (Su et al. 2005) (Li et al. 2017), and the relationship between anti-angiogenic activity and teratogenic effects in development chicken embryos included the primary types of twisting in the spinal cord which caused chicken developmental delay (Beedie et al. 2016). Additionally, exposure to genistein at 0.025 to 0.1 mM significantly decreased the survival rate for zebrafish embryos, which might be related to the inhibition of tyrosine kinase and disruption of several ionic channel in the organism (Kim et al. 2009). Interestingly, the lowest TBARs level was found in the quercetin group at 62.48 nmol/g, even lower than the finding of VC (67.87 nmol/g), which might be attributed to the reported potent antioxidant effects of quercetin with decreased levels of oxidative stress markers and increased antioxidant enzyme activities in mice and rat models (Gerin et al. 2016) (Sharma et al. 2013). Quercetin demonstrated lower chicken embryonic mortality and deformation rate in our study compared with the findings of luteolin and genistein, which could be associated with the lowest TBARs levels. In the chicken embryonic assay, even though the high mortality rates of E2 and flavonoids (except quercetin)

were detected in the higher dose treatments, the NMDR was revealed for luteolin and apigenin in developmental indexes and TBARs levels. A significantly decreased REEW level was detected in the 47.7 μg/kg luteolin but not for the 477 μg/kg group. Only the 45 μg/kg (but not 450 μg/kg) apigenin treatment, had a significantly lower LSI%.

4.5.3 Luteolin, apigenin, and quercetin had the sign of mutagenicity by Ames test at low concentrations range.

We determined the mutagenicity on the low exposure range from 5×10^{-5} to 50 pmol/plate of these four flavonoids as few studies evaluated the mutagenic activity of the flavonoids at these exposures. The exposures were easily overlooked but they were possible exposure levels for humans due to low bioavailability of these flavonoids. The revertants number of VC and positive controls in our study were similar to those of previous findings (Mortelmans and Zeiger 2000), showing the reliability of our study. In our Ames test, luteolin, apigenin, and quercetin showed signs of mutagenicity in two or three test strains, but genistein did not demonstrate such a sign. This result partly agreed with the QSRAs simulation results, in which luteolin and quercetin were classified as mutagenicity positive and apigenin and genistein as mutagenicity negative. Our results were also in good agreement with those of previous research that luteolin showed signs of mutagenicity in the TA102 strain at 116.4 and 174.7 nmol/plate, and quercetin exhibited mutagenicity for three strains TA98, TA100, and TA102 at 12.1 to 147.8 nmol/plate (Flavia Aparecida Resende et al.

2012), even though our test levels are much lower. The potential mutagenicity of flavonoids is mainly due to their prooxidant activity, which will produce free radicals, cause DNA damage, and lead to mutagenesis (Eghbaliferiz and Iranshahi 2016). No mutagenic activity was reported for genistein by others when using the incorporation or the preincubation Ames assays at concentrations 10-3333 μg/plate (Michael McClain et al. 2006). Interestingly the NMDR effect was still found in the Ames test for some flavonoids because that the highest MI level was observed at 5×10^{-3} pmol/plate for quercetin in TA102 strain (with S9) among levels ranged from 5×10^{-5} to 50 pmol/plate.

4.5.4 SARs of four flavonoids on three toxicity endpoints revealed some small structural difference with big toxicity impacts of the test flavonoids.

In general, these four phenolic compounds have similar structures with only the difference in the number of hydroxyl groups or location of the B ring; however, they showed different toxicity responses in several endpoints. Quercetin, belonging to the flavonols with the hydroxyl group at position 3 in ring C, exhibited low developmental toxicity, low binding affinity to major hormone receptors (such as ERs and TRs), but high mutagenic activity even at low concentrations. As an essential compound in the isoflavones group, genistein showed a high binding ability to estrogen receptors and other hormone-related receptors, high acute toxicity and developmental toxicity, but low mutagenicity. For the compounds in the flavone group, apigenin and luteolin showed moderate binding affinity in EDC target

receptors, high developmental toxicity, and high risk of mutagenicity. Our findings generally showed similar pattern as reported in previous findings on the correlation between their structures and cytotoxicity. In a previous study, the 3'- hydroxyl substitution in the B ring (R3 position) played an important role in inhibiting growth of HL-60 cells, which led to luteolin be the most cytotoxic flavone among 14 tested flavonoids (Chen et al. 2014). A SAR study of flavonoid's effects on apoptosis of HL-60 cells indicated that the apigenin had a higher potency than quercetin in inducing cellular DNA fragmentation and ROS generation (Wang, Lin-Shiau, and Lin 1999). In our study apigenin showed the highest lipid peroxidation (TBARs) level in fetal chicken livers, and the TBARs values decreased as the number of hydroxyl groups increased in luteolin (B-3') at the higher dosage group or in quercetin (B-3' and C-3) at both dosage groups. Compared to apigenin, luteolin and quercetin have an ortho-dihydroxy structure in the ring B. Number and position of hydroxy groups in polyphenol structures have been reported to play a critical role in the antioxidant activity of flavonoids (Murias et al. 2005; Wu et al. 2006; Storniolo and Moreno 2018). Thus, the lower TBARs value for quercetin groups might be associated with its higher antioxidant capacity. The additional hydroxyl group at C-3 for quercetin showed an effect in reducing oxidative stress in chicken fetal liver. Several studies reported that the presence of a C-2,3 double bond increased the cytotoxic effects, while the presence of the 3-hydroxyl group in the C-ring lowered the cytotoxicity (Rusak, Gutzeit, and Müller 2005; Menezes et al. 2016). In our study, all four flavonoids had the C-2,3 double bond and quercetin had a 3-hydroxyl group in the C-

ring. Indeed, quercetin with the C-3 hydroxyl group contributed to the lower developmental toxicity in chicken embryos and lower acute rat toxicity from the T.E.S.T. simulation. Our work demonstrated that small structural differences could have big impacts on different toxicity endpoints, and further studies are needed to have a more comprehensive understanding of the impacts of different structure changes on various toxicities. The absorption and metabolism of flavonoids after human consumption also need to be considered to understand the contribution of bioavailability to their underlying toxicology mechanisms. These four flavonoids (luteolin, apigenin, quercetin, and genistein) possessed a similar pharmacokinetics in both animals and humans, and they were extensively metabolized. Different phase II metabolites have been detected in plasma and urine samples in humans, among them glucuronides and sulfates were two primary types (Almeida et al. 2018; Hostetler, Ralston, and Schwartz 2017b;Gu et al. 2006). It is important in the future to evaluate the toxicities of these glucuronides and sulfates.

4.6 Conclusion

Our study applied a new multitiered method consisting of *in silico*, *in vitro*, and *in vivo* tests to estimate the potential toxicity of four common flavonoids (luteolin, apigenin, quercetin, and genistein) effectively. The results indicated that except for their therapeutic potential and chemoprotective ability, they demonstrated toxicity concerns, including developmental toxicity, endocrine disruption, and genotoxicity. The toxic concerns are especially big when young populations are exposed to the

chemicals. In addition, the two *in silico* simulations, molecular docking and T.E.S.T., could provide insightful information in assessing endocrine disrupting activity, acute toxicity, developmental toxicity, and mutagenicity of phenolic compounds. High binding affinity was observed for these four compounds to AR, ER, GR, MR, and TR in the molecular docking. The order of binding affinity to ERs was consistent with the EA results found in MCF-7 cell proliferation assay. The T.E.S.T. simulation results agreed well with the findings from the chicken embryo model and bacterial reverse mutation test. The SAR results showed that genistein (isoflavone) possessed high developmental toxicity and EA, along with low TBARs and MI levels. For two flavones, luteolin showed higher developmental toxicity and signs of genotoxicity than apigenin. Quercetin (flavonol) with 2-hydroxyl substitution in the C ring had a lower developmental toxicity and EA among the test flavonoids. Our approach can be used as valuable alternative toxicity assessment platform for natural compounds, following the guiding principles published by the Society of Toxicology to use alternative ways to reduce animal number, and refine or replace whole animals.

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Author Contribution Statement

Xinwen Zhang: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft. Changqing Wu: Conceptualization, Methodology, Data curation, Supervision, Writing - review & editing, Project administration.

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Chapter 5

THE IMPACT OF DIFFERENTIAL LIGNIN S/G RATIOS ON MUTAGENICITY AND CHICKEN EMBRYONIC TOXICITY

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5.1 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 multitiered method consisted of in silico simulation, in vitro Ames test, and in vivo chicken embryonic assay was employed. In the Ames test, syringol showed signs mutagenicity, whereas 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.

KEYWORDS: Developmental toxicity, Guaiacol, In silico simulation, Lignin, Mutagenicity, Syringol

5.2 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 LB 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 et al., 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 et al., 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 et al., 1999). The component proportion and structure of lignin vary depending on the plant species and environmental factors. In hardwoods, lignin consists of S units and G units (guaiacyl–syringyl lignin), whereas 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 (Santos et al., 2012). Compared with softwood lignin, a greater variance of lignin structure among different species and a higher level of erythro form have 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 because 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 & 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 et al., 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 are 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 (LD50) of animals tested (Russo et al., 2019).

The acute toxicity values of rat LD50 for guaiacol and syringol are 520 and 550 mg/kg, respectively (Orłowski & Boruszak, 1991). However, other toxicology aspects of these significant lignin components are less well understood. 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 biobased 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 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 multitiered 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 0.001–1 mM) and chicken embryonic assay (at 41.3–513 μg/kg), respectively. These test dosages were chosen based on potential exposure level for bisphenol A and other fossil fuel-

based polymer materials, because syringol and guaiacol were good building blocks for bio-based acrylates and polymer production (Erler & 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 end points of varying S/G ratios by the multitiered 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.

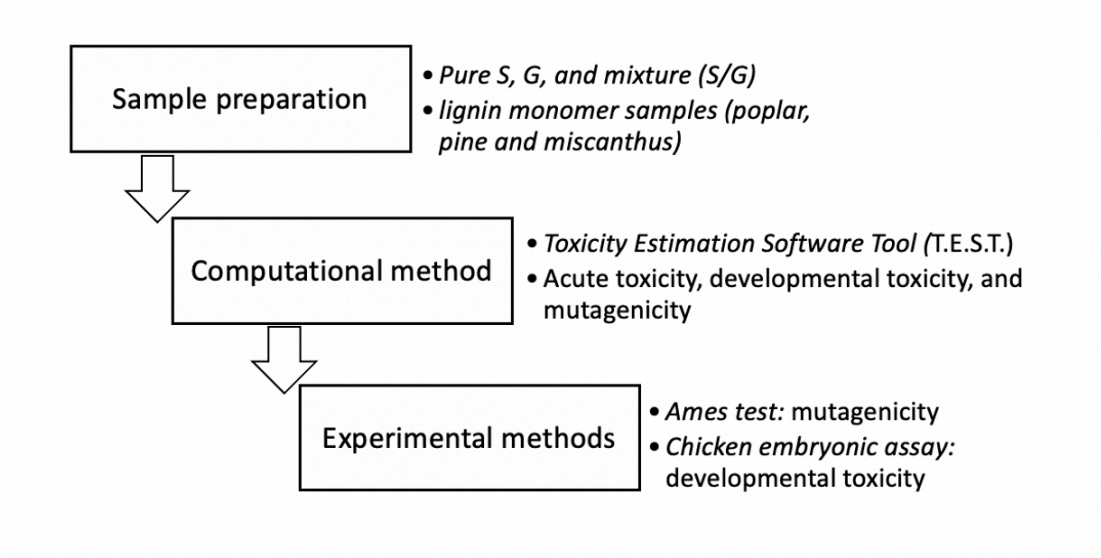

Figure 5.1: Scheme showing multitiered toxicology evaluation of S/G mixture and lignin monomers.

5.3 Methods

5.3.1 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 tree lignin monomers are as below based on the tree genus: pine (*Pinus* spp.): 140 g/mol, miscanthus (MC, *Miscanthus* spp): 140 g/mol, poplar (*Populus* spp.): 146 g/mol.

5.3.2 Preparation and characterization of lignin monomer samples

5.3.2.1 Reductive catalytic fractionation (RCF) of herbaceous lignin

A 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.

5.3.2.2 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 extractions, the monomers were recovered following evaporation of cyclohexane.

5.3.2.3 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).

5.3.3 *Toxicity Estimation Software Tool (T.E.S.T.)*

The Toxicity Estimation Software Tool (T.E.S.T.) was developed by the 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.

5.3.4 *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 exposed 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". 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 was added to the mixture and incubated at 37 °C. After 30 min incubation, 2 mL of top agar was added to each tube and mixed well. The mixture was poured onto 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 in duplicate for each trial.

5.3.5 *Chicken embryonic assay*

5.3.5.1 *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 has 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 tree 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 weight was 60 g) 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 into the egg incubator at 38 ℃ and 60% relative humidity.

5.3.5.2 *Developmental toxicity* e*valuation*

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 reflects the health indicator after embryo exposure to the environmental contaminant. 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.

5.3.6 Data analysis

The results were analyzed with the statistical software package JMP (JMP PRO 15). For 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.

5.4 Results

5.4.1 Guaiacol has a distinguished toxicity profile when compared with the findings for 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 5.1, 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 lower developmental toxicity.

Table 5.1: 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

5.4.2 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 5.2 and 5.3, 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 TA 102 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 5.1). 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 5.3, 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 for the TA 98 strain. After the 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 ($p < 0.05$).

Table 5.2: 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).

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)

0.1% DMSO in PBS was used as negative control and used for dissolving test chemicals. 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. [#] indicates the Significant difference between S/G=0.5 and S/G=2 groups. Data are 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 5.3 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.

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)

0.1% DMSO in PBS was used as negative control and used for dissolving test chemicals. 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. [#] indicates 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).

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

Table 5.4 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 doses 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% stunted 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 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 tree 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 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 tree samples.

Table 5.4 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).

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)

Syrinyol	1	413	8	12.5% (1)	25% (2)
	0.1	51.3	8	12.5% (1)	0.0% (0)
S/G=0.5	1	513	8	0.0% (0)	0.0% (0)
	0.1	44.6	8	12.5% (1)	12.5% (1)
S/G=1	1	446	12	8.3% (1)	0.0% (0)
	0.1	46.3	8	0.0% (0)	0.0% (0)
S/G=2	1	463	12	8.3% (1)	0.0% (0)
	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)

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 is summarized from the data in two trials. The number in parentheses represents the number of dead chicken embryo or malformation chicken embryo.

As shown in Table 5.5, 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 contaminants 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 (Figure 5.2). 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 in syringol (Table 5.1, 5.4, and 5.5, Figure 5.2), pine samples showed the highest MDA when compared with the findings from the other two tree 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 5.4) among all three tested tree lignin monomers.

Table 5.5 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).

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
Syrinyol	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

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. 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.

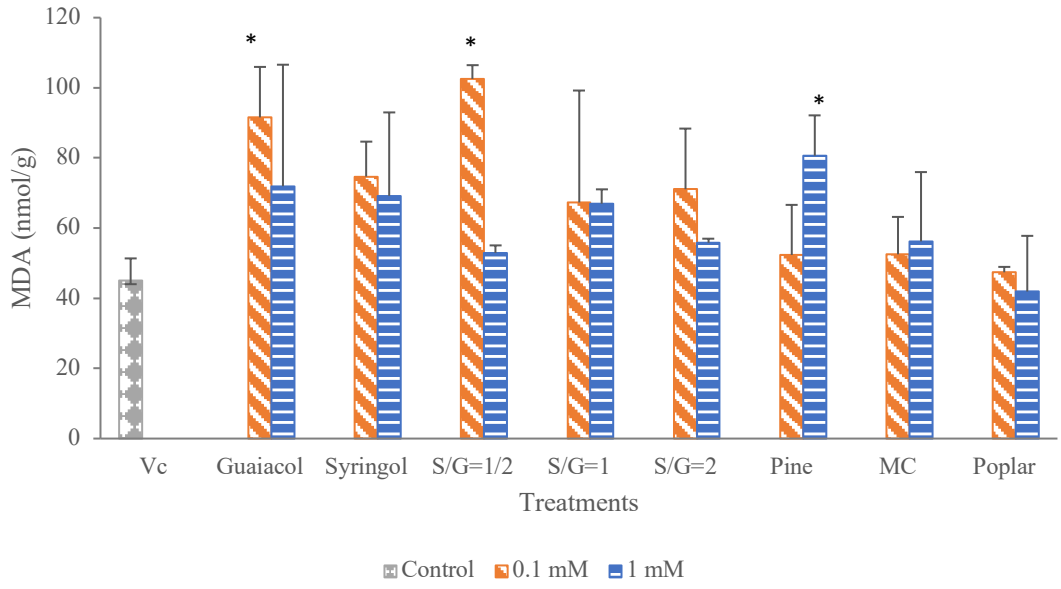

Figure 5.2: Impacts of Guaiacol, Syringol, three ratios of S/G mixture (S/G=0.2, S/G=1, S/G=2) and three lignin monomers (pine, MC, and poplar) on malondialdehyde (MDA) of livers in chicken embryos.

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

5.5 Discussion

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

Besides serving as precursors of plastic polymers, the methoxyphenols (MPs) are 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, are 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 $\mu g/m^3$ (Dills et al., 2006). However, there is 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 testing according to European and American legislation, respectively (Pflieger & Kroflič, 2017). Furthermore, belonging to the MPs, syringol has one more methoxy group them guaiacol, which might contribute 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. Specifically, compared with guaiacol, two 4-substituted guaiacols, creosol (4-methylguaiacol) and 4-ethylguaiacol (4-EG) had higher toxicity for 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 tree 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 5.1). 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 an even lower value than the 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 syringol, while the difference of the oral rat LD50 between guaiacol and syringol was bigger from the T.E.S.T. simulation. Regarding mutagenicity, syringol showed a higher mutagenicity value and belongs to the mutagenicity positive, while guaiacol belongs to the mutagenicity negative. The finding of higher potential mutagenicity of syringol than guaiacol from the *in vitro* Ames test is 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 as suggested by Kato et al. (2018). 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 developmental toxicity, the 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 on chicken embryogenesis were observed after exposure to both guaiacol and syringol at two doses. A 12.5% death rate was 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 the eggs 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 a higher deformation rate for the higher dose group. Additionally, a higher mortality rate was observed in the lower dose of syringol group, which indicates the presence of non-

monotonic dose response (NMDR) effects (Vandenberg et al., 2012). The NMDR included different shapes of the dose-response curves than the traditional one, which means that the lower dose might have a larger effect than the higher dose.

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

Lignin-based polymer materials have been increasingly applied as alternative green materials (Kai et al., 2016; C. Wang, Kelley, & Venditti, 2016). However, toxicology data is limited to single hazard components present in lignin, and the assessment of a set of toxicity properties is absent, which includes different aspects (acute toxicity, mutagenicity, and developmental toxicity). Poplar, pine and miscanthus are promising lignocellulosic feedstocks for 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 the *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, 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 highest 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 the 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 a 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 mutagenicity 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. For our future extensive mutagenicity studies, we will use the recommendation from the updated OECD TG 471 (OECD, 2020) for the compounds or mixtures 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.

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

The developmental toxicity of phenolic compounds has been reported for 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. 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 the S/G = 2 group at 25%. Additionally, a 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 changed 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 for 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 were not determined for the 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, big a variation of S/G ratio existed between the two tree samples, with S/G = 0.067 for pine and S/G = 0.76 for Miscanthus. Both pine and Miscanthus showed a high mortality rate at one test dose at 25%. In addition, a 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 produced similar mortality and deformation rates in the chicken embryonic assay. Clearly, no apparent associations are seen between the S/G ratios (or the mixture concentration) and the chicken developmental toxicities for the three tree monomers, and more research is 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 is widely understood, the toxicology effects of the different structure of monolignols have rarely been studied (Ayyachamy, Cliffe, Coyne, Collier, & Tuohy, 2013). Toxicity studies of these monolignols are important especially after biosourced polyphenols are 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.

5.6 Conclusion

Overall, our study revealed that 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 changes in the developmental indexes and an 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, 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 of 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 to demonstrate the safety concerns 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.

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.

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Chapter 6

REDUCED GENOTOXICITY OF LIGNIN-DERIVABLE REPLACEMENTS TO BISPHENOL A STUDIED USING IN SILICO, IN VITRO, AND IN VIVO METHODS

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6.1 Abstract

Bisguaiacols, lignin‐derivable bisphenols, are considered promising and possibly safer alternatives to bisphenol A (BPA), but comprehensive toxicity investigations are needed to ensure safety. Most toxicity studies of BPA and its analogues have focused on potential estrogenic activity, and only limited toxicological data are available on other toxicity aspects, such as genotoxicity at low exposure levels. In this study, the genotoxicity of six lignin-derivable bisguaiacols with varying regioisomer contents and degrees of methoxy substitution was investigated using a multi-tiered method, consisting of in silico simulations, in vitro Ames tests, and in vivo comet tests. The toxicity estimation software tool, an application that predicts toxicity of chemicals using quantitative structure-activity relationships, calculated that the majority of the lignin-derivable bisguaiacols were non-mutagenic. These results were supported by Ames tests using five tester strains (TA98, TA100, TA102, TA1535, and TA1537) at concentrations ranging from 0.5 pmol/plate to 5 nmol/plate. The potential genotoxicity of bisguaiacols was further evaluated using in vivo comet

testing in fetal chicken livers, and in addition to the standard alkaline comet assay, the formamidopyrimidine DNA glycosylase enzyme-modified comet assay was employed to investigate oxidative DNA damage in the liver samples. The oxidative stress analyses indicated that the majority of lignin-derivable analogues showed no signs of mutagenicity (mutagenic index < 1.5) or genotoxicity, in comparison to BPA and bisphenol F, likely due to the methoxy groups on the lignin-derivable aromatics. These findings reinforce the potential of lignin-derivable bisphenols as safer alternatives to BPA.

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

6.2 Introduction

Bisphenol A (BPA) is an important building block in an array of polymeric systems, such as polycarbonates, polysulfones, epoxy resins, *etc.*, (Mahajan et al. 2020; Trullemans et al. 2021; O'Dea, Willie, and Epps, III 2020), and global demand for BPA is increasing (Lehmler et al. 2018). BPA-based polymers are major constituents in food contact materials due to their chemical inertness, hydrolytic stability, mechanical strength, and high-temperature stability (Mahajan et al. 2020; Trullemans et al. 2021; O'Dea, Willie, and Epps, III 2020; Liguori, Moreno-Marrodan, and Barbaro 2020); however, BPA can potentially leach out from these polymeric products into foodstuffs and be ingested by humans (Rowell, Kuiper, and Preud'Homme 2016). In addition to food contact materials, BPA can be present in

other consumer and industrial products, such as thermal paper and medical materials (Bernier and Vandenberg 2017; Mikołajewska, Stragierowicz, and Gromadzińska 2015). These broad applications allow BPA to distribute across the environment in water, sediment, food, humans, and other animals (Graziani, Carreras, and Wannaz 2019). Related to this distribution, various toxicological problems related to BPA exposure have been reported, including endocrine disruption, genotoxicity, and developmental toxicity (Ma et al. 2019). Because of these toxicity concerns, BPA usage has been restricted in various infant-related products and thermal paper in the United States, Canada, and the European Union (Lehmler et al. 2018; Lee et al. 2021).

To address the adverse health effects surrounding BPA exposure, the use of commercial (petroleum-derived) BPA analogues, such as bisphenol F (BPF), bisphenol S (BPS), bisphenol AF (BPAF), *etc*., has been increasing. However, these bisphenolic replacements have similar chemical structures to BPA, which has led to concerns about their toxicological profiles, such as their endocrine disruption ability (Cabaton et al. 2009; Lei et al. 2019; Le Fol et al. 2017). For example, the estrogenic activity (EA) and anti-androgenic activity of BPF, BPAF, and BPS have been demonstrated in different cell lines (Lei et al. 2019; Le Fol et al. 2017). Potential genotoxicity is also a concern, as it has been reported that two BPA analogues (bisphenol B and BPF) can induce reactive oxygen species (ROS) overproduction, lipid peroxidation, and DNA damage in human peripheral blood cells (Ikhlas, Usman, and Ahmad 2019). Furthermore, BPF and BPAF exert higher DNA double-strand breaks than BPA in human HepG2 cells when assessed by a γ-H2AX assay (Hercog et

al. 2019). Taken together, these toxicology concerns regarding petroleum-derived BPA alternatives provide motivation for the investigation of less toxic, sustainable alternatives to BPA and its commercial analogues.

Recently, lignocellulosic biomass has been viewed as a promising feedstock for the synthesis of bio-based polymeric alternatives to conventional petroleum-based macromolecules (Nicastro, Kloxin, and Epps, III 2018). As a major component of lignocellulosic biomass, lignin is the most abundant and renewable source of potential natural aromatic chemicals (Over et al. 2017). Hence, lignin-derivable polymers have been regarded as greener and more sustainable materials, capable of reducing carbon dioxide and other greenhouse gas emissions (Bass and Epps, III 2021). In addition to sustainability considerations, the thermomechanical properties (*e.g.,* glass transition temperature, Young's modulus, tensile strength) of lignin-derivable polymers are comparable to relevant petroleum-based polymers in laboratory testing (Holmberg et al. 2014; Hambleton and Stanzione III 2021; Pellis et al. 2019).

Bisguaiacols are one key lignin-derivable building blocks and can be readily produced from lignin deconstruction products such as hydroxybenzyl alcohols *(e.g.,* vanillyl alcohol or syringyl alcohol) and methoxyphenols (*e.g.,* phenol, guaiacol, or syringol) (Koelewijn et al. 2019). Furthermore, various regioisomers, such as *p,p′, m,p′,* and *o,p′*-bisguaiacols (see Figure 6.1), can be synthesized in an analogous manner to isomers of BPA and BPF (Nicastro, Kloxin, and Epps, III 2018). A key difference is that bisguaiacols bear methylene bridges present in BPF but are produced without formaldehyde, whereas BPA has an isopropylene bridge (Hernandez et al.

2016). Additionally, in comparison to BPF, bisguaiacol P (BGP) has one methoxy group, bisguaiacol S (BGS) has three methoxy groups, and bisguaiacol M (BGM) has four methoxy groups on the aromatic rings. It has been reported that the methoxy substituents on these bisguaiacols may mitigate the toxicity concerns that are commonly associated with BPA or BPF (Wu and Peng 2018; Amitrano et al. 2021).

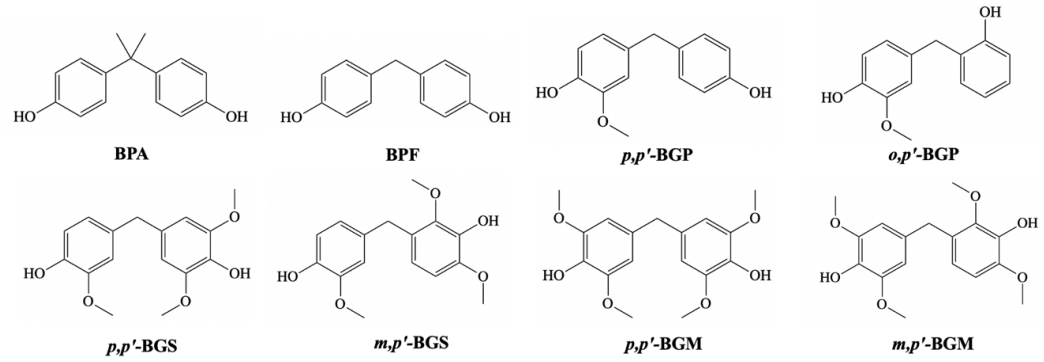

Figure 6.1: Chemical structures of BPA, BPF, *p,p′*-BGP, *o,p′*-BGP, *p,p′*-BGS, *m,p′*-BGS, *p,p′*-BGM, and *m,p′*-BGM.

Previous studies have reported that the six lignin-derivable bisguaiacols had undetectable EA in comparison to BPA and BPF at environmentally relevant concentrations (10^{-10} – 10^{-7} M) (Peng et al. 2020); however, EA is not the only potential toxicity endpoint for these bisguaiacols. Considering the structural similarities between bisguaiacols and BPA/BPF, the possible genotoxicity of these new compounds should not be ignored. *In vivo* assays are one option to estimate the genotoxicity of these bisphenols as recommended in the latest guidelines of the Organization for Economic Co-operation and Development (OECD) (OECD 2016). Following the 3Rs (Replacement, Reduction, and Refinement) for using animals in

toxicological studies, the chicken embryo model has been recognized as an alternative to animal studies (Fonseca, da Silva, and de Morais Ribeiro 2021). Thus, in this study, we investigated the genotoxicity of six bisguaiacols (*p,p′*-BGP, *o,p′*-BGP, *m,p′*-BGS, *p,p′*-BGS, *m,p′*-BGM, and *p,p′*-BGM) using *in silico* simulations with the toxicity estimation software tool (T.E.S.T.), *in vitro* Ames tests, and *in vivo* comet assays, for a more comprehensive evaluation of this important toxicity endpoint. The comet assay was performed on isolated liver cells from the chicken embryos after injection of the test compound(s) on three successive days (day 7 - day 9) at 0.01 mM injection concentration, which resulted in final doses of 6.7 to 10.8 μg/kg egg weight (EW)/day. To measure various types of DNA damage, in addition to the standard strand breaks (LLana-Ruiz-Cabello et al. 2014) (Muruzabal, Collins, and Azqueta 2020), we used formamidopyrimidine DNA glycosylase (Fpg) in combination with the standard comet assay to detect oxidatively damaged DNA, especially oxidized purines (Azqueta et al. 2013). The Fpg enzyme has been added to different cell lines to aid in genotoxicity testing (El Yamani et al. 2017); however, the *in vivo* Fpg-modified assay has only been used in mice, rats, and aquatic organisms in the recent literature (Koelewijn et al. 2019) (Jalili et al. 2020) (Pellegri, Gorbi, and Buschini 2020) (Kolarević et al. 2018). The lipid peroxidation levels also were evaluated in chicken fetal liver samples concurrent with the comet assay to determine the potential correlation between liver oxidative stress and genotoxicity.

6.3 Materials and methods

6.3.1 Chemicals and tested compounds

The six lignin-derivable BPA alternatives, *p,p′*-BGP (molecular weight [MW]: 230.2 g/mol), *o,p′*-BGP (MW: 230.2 g/mol), *m,p′*-BGS (MW: 290.2 g/mol), *p,p′*-BGS (MW: 290.2 g/mol), *m,p′*-BGM (MW: 320.2 g/mol), and *p,p′*-BGM (MW: 320.2 g/mol), tested in this work were synthesized in-house according to methods described in the literature (Nicastro, Kloxin, and Epps, III 2018). These bisguaiacols were >99 mol% pure with respect to regioisomer content. The Trevigen Comet Assay Kit (containing LMAgarose, comet slide, lysis solution, and ethylenediamine tetraacetic acid [EDTA]) was purchased from Trevigen Inc. (Gaithersburg, MD), and Fpg protein was obtained from Sigma-Aldrich (F3174). Fpg activity calibration information was used per the supplier: one unit will cleave 50% of 0.5 pmol of double-stranded DNA oligomer substrate (8-oxoguanine−mutated) in 10 min at 25 °C. The five *Salmonella typhimurium* tester strains (TA98, TA100, TA102, TA1535, and TA1537), mitomycin C (MMC), 2-aminofluorene (2AF), 2-nitrofluorene (2NF), sodium azide, 9-aminoacridine, and 2-aminoanthracene used in the Ames test were purchased from Molecular Toxicology Inc (Boone, NC, USA). Dimethylsulfoxide (DMSO, >99.7%), ethyl methanesulfonate (EMS, 99%), potassium bromate ($KBrO_3$, 99%), phosphate-buffered saline (PBS), Hank's balanced salt solution (HBSS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 99%), bovine serum albumin (BSA), potassium chloride (KCl, >99%), and sodium hydroxide (NaOH, >95 %) were

purchased from Fisher Scientific (Waltham, MA, USA). All chemicals were used as received without further purification.

6.3.2 T.E.S.T.

The United States Environmental Protection Agency (U.S. EPA) developed T.E.S.T. (4.2.1) for users to estimate the toxicity and physical properties of chemicals using a variety of Quantitative Structure-Activity Relationship (QSAR) methodologies, including hierarchical clustering, single model, group contribution, and nearest neighbor approaches (Zhang et al. 2021). Herein, the mutagenicity results were predicted using the consensus method, which averages the predicted toxicity values from two QSAR models (hierarchical clustering and nearest neighbor) with the highest accuracy (Ao et al. 2019). The structures of the test compounds (shown in Figure 6.1) were input into the software using the structure drawing tool. After selecting mutagenicity as the toxicity endpoint and consensus method from the QSAR list, the analysis was initiated by clicking the calculate command, and a predicted result report was generated.

6.3.3 Ames test

The Ames test was conducted with a preincubation method described in OECD guideline 471 (2020) with modifications on the exposure concentrations, with consideration of non-monotonic dose-response and potential human exposure levels of BPA in the environment (OECD 2020). Five *Salmonella typhimurium* tester strains

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

Both negative and positive controls were included in each assay. The negative control was 0.1% DMSO in DI water, and the positive controls were 2NF at 20 μg/plate for TA98 without S9, sodium azide at 1 μg/plate for TA100 and TA1535 without S9, MMC at 2.5 μg/plate for TA102 without S9, 9-aminoacridine at 50 μg/plate for TA1537 without S9, 2AF at 20 μg/plate for TA98, TA100, and TA102 with S9, and 2-aminoanthracene at 2 μg/plate for TA1535 and TA1537 with S9.

6.3.4 Comet assay

6.3.4.1 Standard alkaline comet assay

The standard alkaline comet assay using chicken fetal livers was performed according to Williams et al. (Williams et al. 2014), following the principles of OECD guideline 489 (2016). Fertilized Leghorn eggs were obtained from the University of Delaware (UD) research farm and were injected with BPA, BPF, and the six bisguaiacols at day 7 – day 9 before returning to incubation at 37 °C. The incubation of eggs was terminated on day 9, 3 h after the third injection. Four eggs were used for injection of each compound in every test. Livers from two randomly selected chicken embryos were pooled and processed using the following procedure. One slide with two replicates was used for each pooled liver sample. Livers were collected and rinsed with cold HBSS on ice. After the rinse, livers were transferred to 1 mL of cold HBSS containing 20 mM EDTA and 10 vol% DMSO at pH 7.5, minced into fine pieces by dissecting scissors, and allowed to settle. The cell pellets were collected after 5 min of

centrifugation at 1000 rpm and dissolved in 1× PBS. Next, the cell suspensions were processed with a Trevigen Comet Assay Kit (Trevigen) according to the manufacturer's instructions (Swain and Rao 2011). Briefly, the cell suspensions were mixed with molten LMAgarose and spread onto a comet slide. After the gels were solidified, the slides were placed in lysis buffer for 14 – 16 h. Following lysis, the slides were introduced to an alkaline unwinding solution (200 mM NaOH, 1 mM EDTA, pH >13) for 1 h at 4 °C, and electrophoresed in alkaline electrophoresis solution (200 mM NaOH, 1 mM EDTA, pH >13) for 30 min at 25 V, 4 °C. Then, the slides were immersed twice in DI water (5 min each) followed by 70 vol% ethanol (5 min), and dried at 37 °C for 15 min. Finally, the slides were stained with SYBR Gold for 30 min in the dark and dried completely. The DNA migration on each slide was imaged using a Dragonfly microscope (Dragonfly Spinning Disk and Super Resolution Microscope) in the Bio-imaging Center at UD. The percentages of tail DNA in each image were determined using the CaspLab software (1.2.3beta2), counting >100 cells total (Osipov et al. 2014).

6.3.4.2 Enzyme-modified comet assay

The Fpg enzyme-modified comet assay was used to assess the oxidative DNA damage, especially the 8-hydroxyguanine and ring-opened purines (Dušinská and Collins 1996). All the previous steps were the same as the standard alkaline assay until lysis for 14 – 16 h. Following lysis, the slides were washed three times in enzyme buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8) for 5 min

each. Then, the slides were treated with Fpg enzyme or enzyme buffer (30 μL) and placed in a moisture box at 37 °C for 45 min. The Fpg stock was diluted with enzyme buffer to 0.5 μg/mL. After enzyme treatment, the slides were placed in cold alkaline unwinding solution, and all remaining steps were as described in standard comet assay. The net Fpg-sensitive sites were calculated by subtracting the tail DNA% of the enzyme buffer treatment from the tail DNA% of Fpg enzyme-treated groups.

6.3.5 Thiobarbituric acid reactive substance assay (TBARS assay)

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

6.3.6 Statistical analysis

For the Ames test, the data were presented as the mean ± standard deviation (SD) of two independent tests with two replicates for each concentration. Statistical

analysis was performed on a number of revertants for each strain at different compound concentrations using one-way analysis of variance (ANOVA) followed by Tukey's test in the statistical software package, JMP (JMP PRO 15) (Sall et al. 2017). The mutagenic index (MI) was calculated as the number of revertants for the treatment sample per number of revertants for the negative control of the same strain. A mutagenic positive was identified when a two-fold increase of mutants (MI $\geq$ 2) was detected in at least one concentration with a dose-response relationship. Any compound with statistical significance ($p < 0.05$) as determined by its number of revertants versus the corresponding negative control, but without an MI value higher than 2.0, was defined as having a sign of mutagenicity (X. Zhang, Peng, and Wu 2021). The graphs of these results were made by Prism GraphPad 8 software. For the comet assay and MDA results, data were calculated as mean ± SD of two independent tests with two replicates, the statistical analysis of differences among groups was assessed using one-way ANOVA followed by Dunnett's method (comparison with a control) in JMP.

6.4 Results and discussion

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

We first used *in silico* simulation software to evaluate the suspected mutagenicity of BPA, BPF, and the six bisguaiacols. T.E.S.T. is a toxicity simulation software developed by the U.S. EPA to generate estimates by compiling information from several QSAR models, and it has been widely employed to predict oral toxicity

and mutagenicity of numerous compounds (Dou et al. 2020; Dang et al. 2020). For mutagenicity, the potential toxicity score can range from 0 (non-mutagen) to 1 (mutagen) (US EPA 2020). Table 6.1 summarizes the predicted mutagenicity values of test compounds using the consensus method, in which the average predicted value from all QSAR methodologies was calculated. Chemicals with mutagenicity scores greater than or equal to 0.5 were regarded as mutagenic positive, and compounds with scores less than 0.5 were classified as mutagenic negative by the software (US EPA 2020). BPA showed the lowest mutagenicity value of 0.15, followed by *o,p′*-BGP with the mutagenicity score of 0.16, whereas *m,p′*-BGS possessed the highest value of 0.72. BPF and the remaining other bisguaiacols had mutagenicity values between 0.33 and 0.49. Thus, among the eight test compounds, all lignin-derivable bisguaiacols were classified as non-mutagenic, except *m,p′*-BGS with three methoxy groups (value > 0.5) (Table 6.1). For each pair of stereoisomers, *m,p′* had a higher mutagenicity value than *p,p′*, and *o,p′* had a lower value than *p,p′*. However, because the accuracy of T.E.S.T. simulation results is generally ~80% (Bakhtyari et al. 2013), and because the simulation does not evaluate a dose response, further *in vitro* and *in vivo* experiments were conducted to supplement the *in silico* information.

Table 6.1: Number of methoxy groups and mutagenicity of BPA, BPF, and six bisguaiacol regioisomers simulated by T.E.S.T.

Compounds	Number of Methoxy groups	Mutagenicity	
		Value	Result

BPA	0	0.15	Negative
BPF	0	0.33	Negative
p,p′-BGP	1	0.33	Negative
o,p′-BGP	1	0.16	Negative
p,p′-BGS	3	0.42	Negative
m,p′-BGS	3	0.72	Positive
p,p′-BGM	4	0.35	Negative
m,p′-BGM	4	0.49	Negative

6.4.2 Mutagenicity assessment of six bisguaiacols by the Ames test

To confirm the *in silico* simulation outcomes, we investigated the mutagenic activity of the six bisguaiacols (at treatment concentrations: 10^{-8} to 10^{-4} M; final dosages: 0.5 pmol/plate to 5 nmol/plate) on five tester strains (TA98, TA100, TA102, TA1535, and TA1537). Even though there was a recommendation of using TA98 and TA100 alone to replace the traditional Ames test from the International Workshop on Genotoxicity Testing (R. V Williams et al. 2019), the recently updated OECD TG 471 guideline still suggests using at least five tester strains. Therefore, by following this updated guideline, we included five tester strains in the current study to target frameshift (TA98 and TA1537), base-pair substitution (TA100 and TA1535), and transversion on nucleotides AT (TA102) (Vijay et al. 2018). The positive control of each tester strain, with or without S9 mix, produced a statistically significant increase in the number of revertants colonies vs. the negative controls (Table 6.2 and 6.3). Additionally, the revertants number of the negative controls on five tester strains were within our historical ranges, which confirmed the sensitivity and accuracy of the test system. Finally, no precipitation was detected in the Ames test at the experimental

conditions employed. On the basis of the literature (Resende et al. 2012), the MI value of 2.0 was considered the critical value to determine if a test chemical was mutagenic.

Table 6.2: Mutagenic effects of six bisguaiacols as determined by the Ames test without S9 mix.

Compounds	Concentration (pmol/plate)	Final dosage (× 10^{-6} mg/plate)	Without S9 activation									
			TA1535		TA1537		TA98		TA100		TA102	
			revertants	MI	revertants	MI	revertants	MI	revertants	MI	revertants	MI
o,p'-BGP	5000	1150	9 ± 1	0.9	5 ± 1	0.7	10 ± 1	0.7	77 ± 5	1.1	233 ± 10	0.9
	500	115	12 ± 2	1.2	5 ± 2	0.7	11 ± 3	0.8	72 ± 4	1	240 ± 6	1
	50	11.5	7 ± 2	0.7	6 ± 3	0.9	13 ± 4	0.9	75 ± 4	1.1	250 ± 8	1
	5	1.15	10 ± 2	1	6 ± 1	0.9	16 ± 1	1.1	71 ± 5	1	257 ± 2	1
	0.5	0.115	8 ± 1	0.8	7 ± 1	1	17 ± 2	1.2	69 ± 1	1	257 ± 4	1
p,p'-BGP	5000	1150	8 ± 1	0.8	7 ± 2	1	13 ± 2	0.9	67 ± 2	1	224 ± 6	0.9
	500	115	8 ± 3	0.8	7 ± 4	1.1	16 ± 1	1.1	66 ± 2	0.9	232 ± 3	0.9
	50	11.5	9 ± 4	0.9	7 ± 4	1.1	15 ± 2	1	73 ± 4	1	232 ± 5	0.9
	5	1.15	5 ± 2	0.5	5 ± 4	0.7	17 ± 1	1.2	71 ± 2	1	234 ± 6	1
	0.5	0.115	7 ± 2	0.7	8 ± 2	1.2	18 ± 2	1.3	73 ± 3	1	242 ± 5	1
p,p'-BGS	5000	1450	6 ± 3	0.6	9 ± 4	1.4	12 ± 3	0.9	76 ± 2	1.1	244 ± 6	1
	500	145	8 ± 2	0.8	6 ± 2	0.8	19 ± 2	1.3	88 ± 6	1.3	253 ± 4	1
	50	14.5	11 ± 2	1.1	7 ± 2	1	14 ± 4	1	75 ± 4	1.1	215 ± 7	0.9
	5	1.45	9 ± 2	0.9	5 ± 3	0.8	19 ± 2	1.3	71 ± 2	1	235 ± 6	1
	0.5	0.145	8 ± 1	0.8	7 ± 2	1	18 ± 1	1.3	72 ± 3	1	263 ± 4	1.1
m,p'-BGS	5000	1450	10 ± 1	1.1	8 ± 4	1.2	23 ± 2	1.6	71 ± 3	1	246 ± 4	1
	500	145	8 ± 1	0.8	6 ± 2	0.8	24 ± 4*	1.7	73 ± 3	1	267 ± 4	1.1
	50	14.5	5 ± 3	0.5	7 ± 4	1.1	22 ± 3	1.6	72 ± 3	1	284 ± 6	1.2
	5	1.45	5 ± 2	0.5	7 ± 1	1	20 ± 3	1.4	88 ± 3	1.3	279 ± 2	1.1
	0.5	0.145	8 ± 1	0.8	6 ± 1	0.8	21 ± 1	1.5	83 ± 4	1.2	252 ± 3	1

	5000	1600	8 ± 4	0.8	9 ± 4	1.3	17 ± 1	1.2	71 ± 1	1	269 ± 13	1.1
	500	160	9 ± 1	0.9	8 ± 5	1.2	18 ± 3	1.3	74 ± 6	1.1	258 ± 11	1
p,p'-BGM	50	16	8 ± 1	0.8	10 ± 5	1.5	18 ± 4	1.3	86 ± 4	1.2	245 ± 7	1
	5	1.6	9 ± 4	0.9	7 ± 1	1	22 ± 2	1.5	77 ± 3	1.1	223 ± 4	0.9
	0.5	0.16	8 ± 1	0.8	7 ± 4	1	23 ± 3	1.6	80 ± 2	1.1	216 ± 8	0.9
	5000	1600	12 ± 1	1.3	7 ± 3	1.1	17 ± 1	1.2	75 ± 3	1.1	261 ± 8	1.1
	500	160	7 ± 3	0.7	7 ± 4	1	18 ± 5	1.3	87 ± 3	1.2	263 ± 4	1.1
m,p'-BGM	50	16	8 ± 2	0.8	9 ± 4	1.3	17 ± 4	1.2	87 ± 1	1.2	238 ± 4	1
	5	1.6	8 ± 3	0.8	6 ± 1	0.9	16 ± 2	1.1	78 ± 1	1.1	234 ± 6	1
	0.5	0.16	8 ± 1	0.8	7 ± 4	1	16 ± 1	1.1	81 ± 4	1.2	225 ± 5	0.9
Negative control[a]			10 ± 2		7 ± 2		14 ± 1		70 ± 3		246 ± 4	
Positive control			466 ± 76[b**]		158 ± 14[c**]		454 ± 8[d**]		775 ± 30[b**]		860 ± 14[e**]	

Revertants are presented as means ± SD from two independent trials with two replicates each. MI is calculated as the number of revertants for treated samples divided by the number of revertants for the negative control. Negative control: **[a]** 0.1 vol% DMSO in DI water; Positive control: **[b]** sodium azide (1 μg/plate), **[c]** 9-aminoacridine (50 μg/plate), **[d]** 2NF (20 μg/plate), **[e]** MMC (2.5 μg/plate). Differences were evaluated using one-way ANOVA followed by Tukey's test, and statistical significance was indicated by $*p < 0.05$ and $**p < 0.01$ in comparison to the negative control.

The number of revertants colonies/plates of each compound, the corresponding MI value on five tester strains at five concentrations, and the negative control (at concentration = 0) without S9 mix are shown in Table 6.2. The revertants of the negative control group (0.1 vol% DMSO in DI water) were 10 ± 2, 7 ± 2, 14 ± 1, 70 ±

3, and 246 ± 4 for TA1535, TA1537, TA98, TA100, and TA102, respectively. The results showed that *o,p′*-BGP, *p,p′*-BGP, *p,p′*-BGS, and *m,p′*-BGM had a similar number of revertants to the negative control *S. typhimurium* strains, with the MI lower than 1.5 in the absence of S9 mix. However, *m,p′*-BGS had a higher MI of 1.7 in the TA98 strain at 0.5 nmol/plate (in the absence of S9 mix), which was statistically significant ($p < 0.05$) in comparison to the negative control. For the tests with S9 mixture (Table 6.3), the revertants of the negative control group (0.1 vol% DMSO in DI water) were 8 ± 1, 17 ± 5, 14 ± 4, 83 ± 4, and 264 ± 6 for TA1535, TA1537, TA98, TA100, and TA102, respectively. The *o,p′*-BGP and *p,p′*-BGP compounds had MI values of 1.4 on TA1535 (at 0.5 pmol/plate to 0.5 nmol/plate) and TA1537 (from 0.5 pmol/plate to 5 nmol/plate), respectively. The *p,p′*-BGS showed the highest MI among the six compounds at 1.8 on TA1535 (at 0.05 nmol/plate), and *m,p′*-BGS possessed an MI of 1.6 for TA98 (at 0.05 nmol/plate). The *p,p′*-BGM exhibited an MI value of 1.5 in TA1535 at 5 pmol/plate. There were no MI values greater than 1.2 for the *m,p′*-BGM. However, no significant difference existed for all compounds in the presence of S9 versus the negative control ($p < 0.05$). A non-monotonic dose response was detected for all compounds in at least one tester strain.

Table 6.3: Mutagenic effects of six bisguaiacols determined by the Ames test with S9 mix.

Compounds	Concentration (pmol/plate)	Final dosage ($\times 10^{-6}$ mg/plate)	With S9 activation									
			TA1535		TA1537		TA98		TA100		TA102	
			revertants	MI	revertants	MI	Revertants	MI	revertants	MI	revertants	MI
	5000	1150	10 ± 3	1.3	20 ± 1	1.2	14 ± 4	1	78 ± 4	0.9	285 ± 4	1.1
	500	115	11 ± 1	1.4	18 ± 1	1.1	17 ± 2	1.2	74 ± 1	0.9	287 ± 4	1.1
o,p′-BGP	50	11.5	11 ± 2	1.3	18 ± 3	1.1	14 ± 5	1	82 ± 3	1	282 ± 6	1.1
	5	1.15	12 ± 2	1.4	19 ± 2	1.1	16 ± 4	1.1	82 ± 2	1	280 ± 4	1.1
	0.5	0.115	9 ± 3	1.1	19 ± 3	1.2	15 ± 2	1	90 ± 3	1.1	274 ± 6	1
	5000	1150	11 ± 2	1.3	17 ± 1	1	17 ± 1	1.2	78 ± 6	0.9	283 ± 3	1.1
	500	115	8 ± 3	1	17 ± 4	1	16 ± 4	1.1	76 ± 4	0.9	276 ± 6	1
p,p′-BGP	50	11.5	10 ± 1	1.3	22 ± 5	1.3	16 ± 3	1.1	79 ± 3	1	270 ± 4	1
	5	1.15	8 ± 3	1	23 ± 5	1.4	15 ± 4	1.1	81 ± 2	1	263 ± 4	1
	0.5	0.115	11 ± 2	1.3	21 ± 1	1.3	17 ± 2	1.2	80 ± 4	1	265 ± 6	1
	5000	1450	12 ± 2	1.4	17 ± 1	1	15 ± 4	1.1	79 ± 3	1	296 ± 3	1.1
	500	145	13 ± 1	1.6	13 ± 2	0.8	20 ± 2	1.4	77 ± 4	0.9	288 ± 4	1.1
p,p′-BGS	50	14.5	15 ± 1	1.8	16 ± 2	0.9	17 ± 5	1.2	81 ± 1	1	306 ± 6	1.2
	5	1.45	12 ± 3	1.5	14 ± 2	0.8	18 ± 3	1.3	82 ± 1	1	274 ± 6	1
	0.5	0.145	12 ± 1	1.5	16 ± 2	0.9	19 ± 3	1.4	77 ± 3	0.9	286 ± 4	1.1
	5000	1450	11 ± 3	1.4	16 ± 3	1	15 ± 6	1.1	83 ± 2	1	269 ± 13	1
	500	145	12 ± 1	1.5	21 ± 4	1.2	20 ± 3	1.4	87 ± 2	1	286 ± 4	1.1
m,p′-BGS	50	14.5	9 ± 2	1.1	18 ± 2	1.1	22 ± 1	1.6	99 ± 5	1.2	293 ± 4	1.1
	5	1.45	11 ± 1	1.3	22 ± 2	1.3	17 ± 3	1.2	96 ± 8	1.2	286 ± 11	1.1
	0.5	0.145	9 ± 3	1.1	20 ± 1	1.2	19 ± 1	1.4	89 ± 3	1.1	291 ± 16	1.1
	5000	1600	10 ± 1	1.3	21 ± 2	1.2	14 ± 5	1	79 ± 1	1	292 ± 16	1.1
	500	160	12 ± 2	1.4	13 ± 1	0.8	16 ± 5	1.1	73 ± 4	0.9	311 ± 13	1.2
p,p′-BGM	50	16	11 ± 3	1.4	15 ± 2	0.9	15 ± 6	1.1	89 ± 3	1.1	288 ± 15	1.1
	5	1.6	12 ± 1	1.5	17 ± 1	1	19 ± 2	1.3	88 ± 3	1.1	270 ± 15	1
	0.5	0.16	11 ± 1	1.4	20 ± 2	1.2	15 ± 4	1.1	86 ± 4	1	260 ± 11	1
	5000	1600	9 ± 3	1.1	20 ± 2	1.2	17 ± 2	1.2	81 ± 4	1	294 ± 6	1.1
	500	160	8 ± 1	1	14 ± 3	0.8	17 ± 2	1.2	78 ± 3	0.9	284 ± 8	1.1
m,p′-BGM	50	16	10 ± 1	1.2	19 ± 1	1.2	15 ± 6	1	88 ± 5	1.1	273 ± 10	1
	5	1.6	10 ± 2	1.2	19 ± 2	1.1	13 ± 3	0.9	85 ± 4	1	265 ± 13	1
	0.5	0.16	9 ± 3	1.1	18 ± 2	1.1	15 ± 4	1	84 ± 3	1	250 ± 4	0.9
Negative control[a]			8 ± 1		17 ± 5		14 ± 4		83 ± 4		264 ± 6	
Positive control			218 ± 11[b**]		389 ± 33[b**]		687 ± 33[c**]		701 ± 30[c**]		869 ± 13[c**]	

Revertants are presented as means ± SD from two independent trials. MI is calculated as the number of revertants for treatment divided by the number of revertants for the negative control. Negative control: [a] 0.1 vol% DMSO in DI water; Positive control: [b] 2-aminoanthracene (2 μg/plate), and [c] 2AF (20 μg/plate). Differences were evaluated using one-way ANOVA followed by Tukey's test, and statistical significance was indicated by $*p < 0.05$ and $**p < 0.01$ in comparison to the negative control.

Our Ames test results suggested no mutagenicity for the six bisguaiacols because none of their MI values reached the critical value of 2.0 (Resende et al. 2012). However, the *m,p′*-BGS showed a sign of mutagenicity with a significantly increased number of revertants compared to the negative control, which was in general agreement with the *in silico* simulation, as only the *m,p′*-BGS was classified as mutagenic positive using the T.E.S.T. software. We did not include the BPA and BPF in the Ames test as there is sufficient published data on their mutagenicity, which indicates a lack of mutagenicity for BPA and BPF in the Ames test (Fic et al. 2013) (Xin et al. 2015). Specifically, non-mutagenic activity was reported for BPA and BPF at 4 - 500 μg/plate in *S. typhimurium* TA98 and TA100 (Fic et al. 2013). No mutagenic activity of BPA was detected in any of the tester strains (TA97, TA98, TA100, TA102, and TA1535) at 10 - 5000 μg/plate (Xin et al. 2015). As the lignin-derivable bisguaiacols possess similar structures to bisphenols, and these bisguaiacols had not been tested for mutagenicity using the Ames test, our findings are the first to

show that six test bisguaiacols are not mutagenic in *S. typhimurium* TA98, TA100, TA102, TA1535, and TA1537 with and without S9 metabolic activation at the tested concentrations.

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

In this study, two comet assays (standard alkaline version and Fpg enzyme-modified) were performed using fetal chicken livers after a three-day (day 7 - day 9) treatment of test compounds at 0.01 mM, resulting in final doses of 7.7 μg/kg EW/day, 6.7 μg/kg EW/day, 7.7 μg/kg EW/day, 9.8 μg/kg EW/day, and 10.8 μg/kg EW/day for BPA, BPF, BGPs (*p,p′* and *o,p′*), BGSs (*p,p′* and *m,p′*), and BGMs (*p,p′* and *m,p′*), respectively. The estimated human equivalent doses were calculated from the final applied doses by considering the human safety factor (÷10) and the human equivalent dose factor from the chickens (× 18.5) (Zhang, Peng, and Wu 2021). These exposure dosages were lower than the reference dose for human of BPA 50 μg/kg body weight (BW)/day established by the U.S. EPA and close to the potential human exposure dosage of BPA (Wang, Liu, and Liu 2017). EMS and $KBrO_3$ (at 1 mM injection concentration) served as positive controls for standard alkaline comet and Fpg enzyme-modified comet assays, respectively. Two negative controls, consisting of a non-treated group and a vehicle control (VC) group (0.1 vol% DMSO), were included for both assays. In the standard alkaline comet assay, the tail DNA% for two negative controls were 6.39 ± 1.90% (non-treated group) and 8.85 ± 0.74% (VC) (shown in

Figure 6.2). On the other hand, EMS showed the highest tail DNA% value (31.67 ± 1.76%) [$p < 0.01$], followed by the BPA (19.09 ± 4.96%) [$p < 0.05$]. The *m,p′*-BGS and *m,p′*-BGM had nearly equivalent tail DNA% values (15.44 ± 5.03% and 15.49 ± 5.49%, respectively). The tail DNA% values for BPF (13.85 ± 2.57%) and *p,p′*-BGS (14.49 ± 2.95%) were comparable. Furthermore, *p,p′*-BGP, *o,p′*-BGP, and *p,p′*-BGM showed mean values (10.45 ± 1.19% - 11.73 ± 4.02%) that were lower than the other compounds.

The tail DNA% results of Fpg-modified comet assay were calculated as net Fpg-sensitive sites by subtracting the tail DNA% of the enzyme buffer treatment from the tail DNA% of Fpg enzyme-treated groups (Figure 6.2). The Fpg protein concentration (at 0.5 μg/mL) applied in this study was in accordance with dose ranges from the literature and the response from the positive control treatment (Guichard et al. 2015) (Chen et al. 2008). The tail DNA% values for BPA (17.93 ± 3.12%) and BPF (16.07 ± 1.20%) were similar to that of the positive control, $KBrO_3$ (19.66 ± 1.77%). Both BPA, BPF, and $KBrO_3$ group showed significantly higher values of Fpg-sensitive sites than the two controls (VC and non-treated group). In contrast, none of the six bisguaiacols showed increased Fpg-sensitive sites as the values of tail DNA% (3.15 ± 2.99% to 5.74 ± 4.14%) were comparable to those of the VC (2.39 ± 2.79%) and non-treated group (2.40 ± 0.46%) [Figure 6.2].

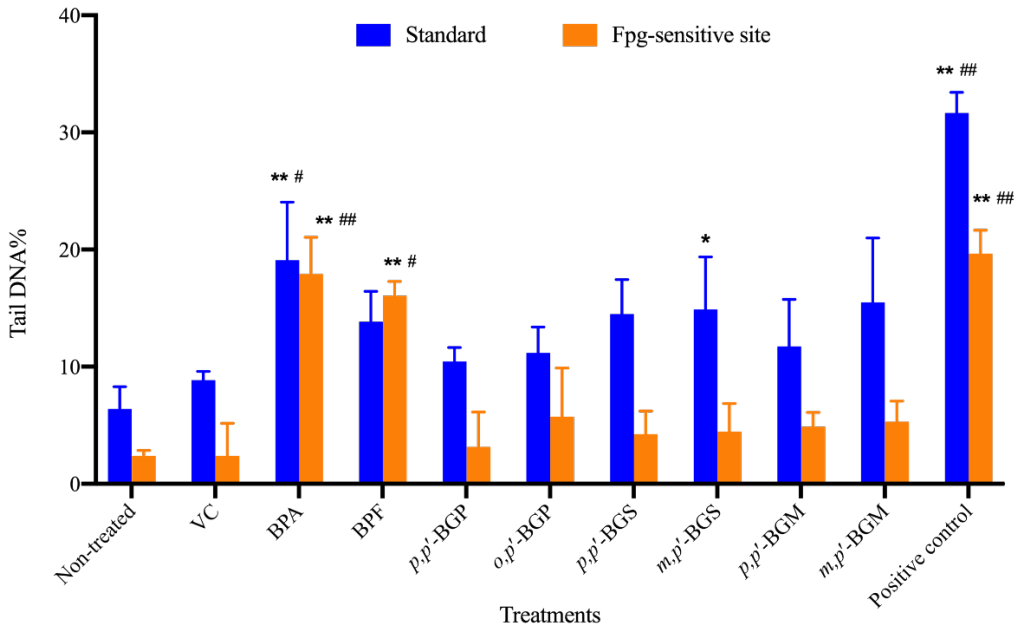

Figure 6.2: Comet assay results (including the standard and Fpg-modified comet assay) after a three-day (day 7 - day 9) treatment of test compounds at 0.01 mM (BPA, BPF, p,p′-BGP, o,p′-BGP, m,p′-BGS, p,p′-BGS, m,p′-BGM, and p,p′-BGM).

Two control groups were included: non-treated and VC groups. The positive control for standard and Fpg-modified comet assay were EMS and $KBrO_3$, respectively. Differences were evaluated using one-way ANOVA and followed by the Dunnett's test. The statistical significance was indicated by $*p < 0.05$ and $**p < 0.01$ in comparison to the non-treated group, and $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus the VC group under each assay condition.

DNA damage is an important initial event in carcinogenesis (Barnes et al. 2018). For the first time, our study assessed DNA damage levels in the chicken fetal

livers by both the standard alkaline and enzyme-modified comet assays. The standard alkaline version assesses DNA lesions that include strand breaks and alkali-labile sites. Adding the Fpg-enzyme treatment to the standard comet assay enables this assay to detect oxidatively damaged DNA. As a DNA repair enzyme, Fpg can convert damaged bases (*e.g.*, oxidized purines) to strand breaks in DNA, and these breaks can be identified in the later procedures of the comet assay. As mentioned above, the highest and significantly increased tail DNA% (strand breaks and alkali-labile sites) was exhibited in the BPA treatment group, followed by *m,p′*-BGS and *m,p′*-BGM, with the order for the rest of the compounds as: *p,p′*-BGS > BPF > *p,p′*- BGP, *o,p′*-BGP, or *p,p′*-BGM. The standard comet assay findings are in agreement with previous genotoxicity studies on BPA and BPF in human cells (Fic et al. 2013) (Chen et al. 2008) (Barnes et al. 2018). BPA at 0.1 μmol/L to 10 μmol/L induced significant DNA damage after a 24 h exposure with no dose-response relationship in the human hepatoma cells (HepG2), whereas BPF did not induce such an increase in DNA damage (Fic et al. 2013). DNA damage also was detected by the *in vivo* alkaline comet assay in liver tissues of female rat offspring at three different pubertal periods after BPA treatment (at 0.5 and 50 mg of BPA/kg), and in thyroid tissue from rats after 35 days of BPA treatment at 200 mg/kg (Chen et al. 2008) (Barnes et al. 2018). Furthermore, the oxidative damage to DNA pyrimidines and purines was identified by the alkaline comet test with DNA repair enzymes (endonuclease III and human 8-oxoguanine DNA glycosylase) after exposure to BPA, BPAF, BPS, and BPF in human

peripheral blood mononuclear cells from 0.001 to 1 mg/mL at two exposure times (4 and 48 h) (Mokra et al. 2018).

Most notably, our study revealed that the six bisguaiacols showed significantly lower levels of oxidative DNA damage in the Fpg-modified comet assay in comparison to BPA and BPF. After Fpg-enzyme treatment, BPA and BPF produced increased oxidative DNA damage, but most of the bisguaiacols showed a comparable number of Fpg-sensitive sites with the VC group at < ~5% tail DNA% (Figure 2). The *o,p'*-BGP treated group had the highest level of Fpg-sensitive sites among the six compounds at 5.74 ± 4.14%, which was still much lower than the BPA (17.93 ± 3.12%) and BPF (16.07 ± 1.20%). The lower oxidative DNA damage of lignin-derivable bisguaiacols versus BPA or BPF is mainly attributed to the methoxy groups on the aromatic rings. It has been demonstrated that natural phenolic compounds and bulk lignin with *ortho*-methoxy groups have electron-donating ability with higher antioxidant activity and DNA damage-protective effects (Mohammed et al. 2020; L.-L. Zhang et al. 2017; Hu, Yuan, and Kitts 2007). Additionally, our study suggested that increasing the number of methoxy groups on the aromatic moieties led to only a slight effect on oxidative DNA damage levels. Thus, there was no major difference between the bisguaiacols with different numbers of methoxy groups on the oxidative DNA damage noted in the comet test. DNA damage detected by the comet assay and gene mutation revealed from the Ames test are both essential genotoxic endpoints but differ from each other. DNA damage is a structural change, usually as single or double-strand breaks, whereas mutation is a change in the nucleotide sequence of

DNA. Therefore, a single test usually is not sufficient to thoroughly investigate the genotoxicity of the test compounds (Sun et al. 2020). In this study, we applied an *in vitro* Ames test and an *in vivo* comet assay to probe gene mutation and DNA damage, respectively. Our results showed that most bisguaiacols did not exhibit any genotoxicity under the applied current experimental conditions.

6.4.4 TBARS assay

During chicken embryo growth, chorioallantoic respiration turns into pulmonary respiration and accelerates oxidative metabolism (Xiao et al. 2016). Increased oxidative stress can lead to numerous disorders and even mortality of chicken embryos (Li et al. 2020). The MDA level, which served as an essential biomarker for oxidative stress, was measured in fetal livers after a three-day treatment (at 0.01 mM) of BPA, BPF, *p,p′*-BGP, *o,p′*-BGP, *m,p′*-BGS, *p,p′*-BGS, *m,p′*-BGM, *p,p′*-BGM, and $KBrO_3$. As shown in Figure 6.3, the VC group and the non-treated group had MDA values of 70.62 ± 8.04 and 70.90 ± 3.04 nmol/g, respectively. The treatment groups showed relatively higher MDA values ranging from 73.23 ± 7.42 to 94.34 ± 22.15 nmol/g, but without a significant difference ($p > 0.05$).

The pro-oxidant activity of BPA and its commercial analogues has been regarded as one critical mechanism that results in adverse effects in humans and other animals. Exposure to BPA at 200 ppm on day 4 induced considerably higher MDA levels in chicken embryos, though the lower BPA concentration (50 ppm) group had no such impact (Gharibi et al. 2013). Substantial levels of MDA and hydrogen

peroxide also have been demonstrated in rats after exposure to BPA at 25 mg/kg/day (Kabuto, Amakawa, and Shishibori 2004) (Avci et al. 2016). In addition to the MDA biomarker, increased oxidative stress and decreased antioxidant enzyme activities were detected in the hepatic tissue of female rat offspring exposed to BPA at 0.5 and 50 mg/kg/day (Eid, Eissa, and El-Ghor 2015). In comparison to other literature (Gharibi et al. 2013; Kabuto, Amakawa, and Shishibori 2004; Avci et al. 2016), our study did not exhibit a significantly increased MDA level in the BPA treatment, which might be attributed to the varying dosage levels and durations in different animal models. Most of the literature reports (Chen et al. 2008; Li et al. 2020; Gharibi et al. 2013) on BPA showed significantly different MDA levels in rats or mice models with much higher dosages (around 25 - 50 mg/kg) than the dose applied in our work (7.7 μg/kg). One of the critical mechanisms of genotoxicity induced by BPA and BPF is the increased cellular oxidative stress and generation of reactive species, especially quinones, during their biotransformation, which react with DNA and cause DNA damage (Hercog et al. 2019; Fic et al. 2013). Although the MDA levels were not significantly different between bisguaiacols and bisphenols (BPA and BPF), other oxidative pathways could be involved in pro-oxidant activities. In the future, additional oxidative stress biomarkers (*e.g.,* ROS, mitochondrial membrane potential, antioxidant enzymes levels) can be included in the chicken embryo model for a more comprehensive evaluation of oxidative stresses and their associations with genotoxicity.

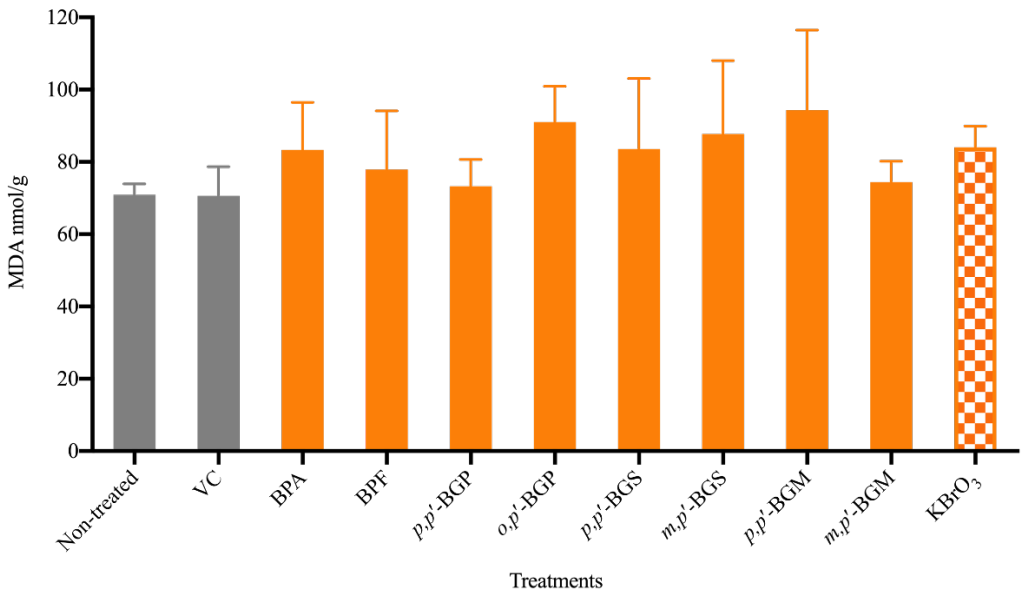

Figure 6.3: MDA values of fetal liver samples on day 9 after treatment with BPA, BPF, p,p′-BGP, o,p′-BGP, m,p′-BGS, p,p′-BGS, m,p′-BGM, p,p′-BGM, and KBrO3.

All values are expressed as mean ± SD from two independent trials.

6.5 Conclusion

In this work, the genotoxicity of six lignin-derivable bisguaiacols, BPA, and BPF was assessed by an *in silico* T.E.S.T. tool, *in vitro* Ames test, and *in vivo* comet assay to probe mutagenicity and DNA damage. First, *in silico* results revealed that all bisguaiacols were non-mutagenic, except for *m,p′*-BGS. Second, *in vitro* Ames test suggested no bisguaiacols had mutagenicity in five tester strains. Building upon those conclusions, two forms of the comet assay (standard alkaline and Fpg enzyme-modified) were applied to chicken embryo models. In the standard alkaline assay,

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

Author Contribution Statement

Xinwen Zhang: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft. Jignesh S. Mahajan: Investigation, Writing - review & editing. LaShanda T. J. Korley: Conceptualization, Writing - review & editing. Thomas H. Epps, III: Conceptualization, Methodology, Writing - review & editing. Changqing Wu: Conceptualization, Methodology, Data curation, Supervision, Writing - review & editing, Project administration.

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Chapter 7

LIGNIN-DERIVABLE MINIMAL DEVELOPMENTAL TOXICITY ALTERNATIVES TO BISPHENOL A WITH POTENTIALLY UNDETECTABLE ESTROGENIC ACTIVITY AND

7.1 Abstract

Lignin-derivable bisguaiacols/bissyringols are viable alternatives to commercial bisphenols; however, many bisguaiacols/bissyringols (*e.g.*, bisguaiacol F [BGF]) have unsubstituted bridging carbons between the aromatic rings, making them more structurally similar to bisphenol F (BPF) than bisphenol A (BPA) – both of which are suspected endocrine disruptors. Herein, we investigated the estrogenic activity (EA) and developmental toxicity of dimethyl-substituted bridging carbon-based lignin-derivable bisphenols (bisguaiacol A [BGA] and bissyringol A [BSA]), as platforms toward safer BPA replacements. Notably, BSA showed undetectable EA at seven test concentrations (from 10^{-12} M to 10^{-6} M) in the MCF-7 (a human breast cancer cell) cell proliferation assay, and BGA had undetectable EA at four test concentrations. Estradiol, a natural estrogen hormone, expectedly had detectable EA at all test concentrations, and BPA had detectable EA at five concentrations (from 10^{-10} M to 10^{-6} M). The undetectable EA for BSA is likely due to the presence of the two methoxy groups on each aromatic ring that may increase steric hindrance around the phenolic hydroxyls and thus reduce interactions with binding pockets on the estrogen

receptors. Additionally, all lignin-derivable compounds showed significantly lower expression fold changes (from ~1.81 to ~4.41) in chicken fetal liver for an estrogen-response gene (apolipoprotein II) in comparison to BPA (fold change of ~11.51), which is indicative of a significantly reduced estrogenic response. Moreover, *in vivo* chicken embryonic assay results revealed that lignin-derivable monomers had minimal developmental toxicity vs. BPA at environmentally relevant test concentrations (8.7 to 116 μg/kg). Altogether, the methoxy substituents on lignin-derivable bisphenols appear to be a positive factor in the development of safer, "EA-free" BPA alternatives.

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

7.2 Introduction

Bisphenol A (BPA) is an essential building block for many polymeric systems, including polycarbonates, polysulfones, and epoxy resins, and these materials are widely used in applications such as food contact materials (FCMs) (Trullemans et al., 2021; Mahajan et al., 2020). However, due to the negative health impacts of BPA (*e.g.,* endocrine disruption, genotoxicity, reproductive toxicity) and increased exposure of humans to BPA, many countries have implemented restrictions on the application of BPA in FCMs, especially in products intended for infants and children (Vom Saal et al., 2012; Ďurovcová et al., 2022; Yin et al., 2017; Chen et al., 2015). For example, the use of BPA in baby bottles was banned in Canada in 2008 (Erler and Novak,

2010), and the European Commission banned BPA usage in coatings of infant-related packaging in 2011 (Usman and Ahmad, 2019). The United States (U.S.) Food and Drug Administration (FDA) took similar steps to ban the use of BPA in infant-associated packaging in 2013 (Usman and Ahmad, 2019). On top of the toxicity concerns, BPA is derived from petrochemical feedstocks, which are limited in quantity, unevenly distributed across the globe, and are associated with environmental concerns (Bass and Epps, III, 2021). Thus, less toxic and sustainable alternatives to BPA are highly desirable.

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

Lignin is the most abundant potential source of renewable aromatic chemicals (Mahajan et al., 2020; Bass and Epps, III, 2021; O'Dea et al., 2020; Schutyser et al., 2018; Cywar et al., 2022; Nicastro et al., 2018). Bulk lignin can be deconstructed into several substituted phenols and can be further converted to bisguaiacols/bissyringols (O'Dea et al., 2020; Schutyser et al., 2018; Mahajan et al., 2020). These lignin-derivable bisphenols are proposed as safer alternatives to commercial bisphenols because the methoxy groups on these bisguaiacols/bissyringols are believed to hinder the binding of phenolic hydroxyls to estrogen receptors (Amitrano et al., 2021). Peng et al. reported that three bisguaiacol F (BGF) mixtures with different regioisomer contents showed lower estrogenic activity (EA) than BPA in two breast cancer (MCF-7) cell-based assays. Apart from BGF, other bisguaiacols (bisguaiacol P [BGP], bisguaiacol S [BGS], and bisguaiacol M [BGM]) with varying degrees of methoxy substitution have demonstrated lower EA, genotoxicity, and oxidative DNA damage when compared to BPA (Peng et al., 2020; Zhang et al., 2022). However, BGF/BGM/BGP/BGS possess an unsubstituted bridging carbon between the aromatic rings, and the absence of substituents on the bridging carbon in these bisguaiacols allows free rotation of the resultant polymer backbone that leads to the lower glass transition temperatures (T_gs) vs. those of BPA analogues. By incorporating dimethyl substituents on the bridging carbon, the T_gs of lignin-derivable polymeric systems can be made comparable to those of the BPA counterparts (Mhatre et al., 2023). Additionally, polymers containing these lignin-derivable building blocks can enhance their mechanical properties. For example, lignin-derivable non-isocyanate

polyurethanes (NIPUs) have been reported with improved toughness relative to petroleum-based NIPUs (Mhatre et al., 2023). Nevertheless, it is equally important to consider the toxicity potential of the dimethyl-substituted bridging carbon-based lignin-derivable bisphenols (bisguaiacol A [BGA] and bissyringol A [BSA]) (Epps, III et al., 2022).

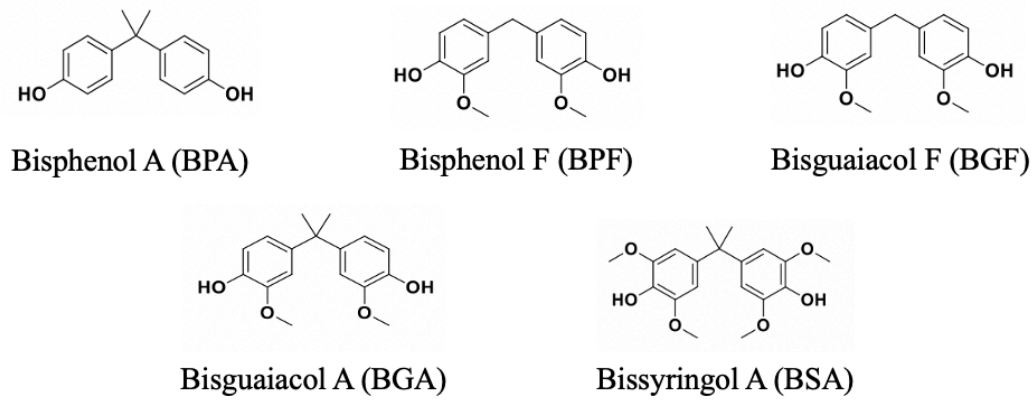

Figure 7.1: Structures of BPA, BPF, BGF, BGA, and BSA.

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

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

7.3 Materials and methods

7.3.1 Chemicals and supplies

BGA (≥99%), BSA (≥99%), and BGF (≥99%) were synthesized according to published literature (Epps, III et al., 2022; Nicastro et al., 2018). BPA (>99%) and BPF (≥99%) were purchased from TCI. 17β-estradiol (E2, ≥98%), dimethyl sulfoxide (DMSO, >99.7%), and phosphate-buffered saline (PBS, 1X) were purchased from Fisher Scientific (Waltham, MA). The MCF-7 human breast cancer cell line was

purchased from the American Type Culture Collection (ATCC No. HTB-22). The Catalase Assay Kit (707002) and thiobarbituric acid reactive substance (TBARS) [TCA Method] Assay Kit (700870) used in the chicken embryonic assay were purchased from Cayman Chemical (MI, USA). The primers (β-actin, ApoII, and VTGII) and Gene Expression Master Mix were purchased from Integrated DNA Technologies, Inc (Coralville, IA). All chemicals were used as received without further purification.

7.3.2 Molecular docking

The endocrine disrupting potentials of E2, BPA, BPF, BGF, BGA, and BSA were assessed on the Docking Interface for Target Systems platform (named endocrine disruptome tool, http://endocrinedisruptome.ki.si) by AutoDock Vina (Kolšek et al., 2014). The ligand structures were generated by ChemSketch to obtain the SMILES (Simplified Molecular-Input Line Entry-System) files as inputs for the endocrine disruptome tool. The binding affinities were predicted between ligands and 14 nuclear receptors, including androgen receptor (AR), estrogen receptors (ERs), glucocorticoid receptor (GR), liver X receptor (LXR), mineralocorticoid receptor (MR), peroxisome proliferator-activated receptor (PPAR), progesterone receptor (PR), retinoid X receptor (RXR), and thyroid receptor (TR). For androgen and estrogen receptors, both agonist and antagonist (an) conformations were included. Three thresholds were set per structure to divide test compounds into four probability binding classes (very

strong binding, strong binding, moderate binding, and weak binding) (Kolšek et al., 2014).

7.3.3 T.E.S.T.

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

7.3.4 *MCF-7 cell proliferation assay*

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

maximum %E2 (%RME2) = 100 × $(OD \text{ of test} - OD\ of\ VC)/(MAX\ OD\ of\ E2 - OD\ of\ VC)$.

7.3.5 Chicken embryonic assay

7.3.5.1 Experiment 1

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

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

mM EDTA, pH 7.0), following the protocol from the Catalase Assay Kit (Cayman Chemical, MI USA).

7.3.5.2 Experiment 2

Fertilized Leghorn eggs (136) were obtained from the UD research farm and were injected with VC, E2, BPF, BGF, BGA, and BSA at two selected dosages (0.01 mM and 0.1 mM injection concentration at 0.2 mL, resulting final dosage shown in Table 2) following the same procedure as described in Experiment 1. On day 18, all embryos were dissected, and the developmental indexes were recorded and calculated as described above.

7.3.5.3 Ribonucleic acid (RNA) extraction and real time-PCR

Eggs used for real time-PCR were injected with the test compounds at 0.1 mM (0.2 mL) one time per day on day 13 and day 15, which was the high dosage used in the chicken embryonic assay and also within the effective range for the target genes (Li et al., 2014). Livers from embryos were collected on day 16 and total RNA was extracted from liver tissues using the RNeasy Plus Mini Kit (QIAGEN, Germantown, MD) following the protocol from the manufacturer. The quality and concentration of RNA were determined using the NanoDrop One instrument (Thermo Scientific). The RNA samples with the ratio of absorbance at 260 nm and 280 nm in the range of 1.9 – 2.2 were used for further steps. Total RNA (1 μg) was reverse transcribed by the QuantiTect Reverse Transcription Kit (QIAGEN, Germantown, MD). Then, real time-

PCR was performed using complementary DNA (2 μL), 2X PrimTime gene expression master mix (10 μL, IDT), and a mixture of primers and probes (2 μL, shown in Table A1) on a BioRad CFX96 Real-Time System at a total reaction volume of 20 μL. We used β-actin as the housekeeping gene, and all primer and probe sequences are listed in Table A1. The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

7.3.6 Statistical analysis

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

7.4 Results

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

The binding affinities of the test compounds (E2, BPA, BPF, BGF, BGA, and BSA) to 14 endocrine-related nuclear receptors were investigated using molecular docking; full data is shown in Table A2. E2 was included as the positive control and showed high-level (very strong) binding affinities to AR (-10.5 kcal/mol), AR an (-10.1 kcal/mol), ER α (-10.6 kcal/mol), ER β (-10.0 kcal/mol), ER β an (-9.2 kcal/mol), and MR (-8.6 kcal/mol) [see Table A2]. Figure 7.2 shows the binding energies of test chemicals to 11 nuclear receptors that exhibited differences between lignin-derivable monomers and the positive control. The dashed line/solid line shown in Figure 2 represented the threshold value for strong-binding ability to each receptor that is generated by the software. As shown in Figure 2, BGA had a comparable binding affinity among the majority of test receptors compared to BPA with the exception of AR and MR, whereas BSA had much lower (weak) binding affinities to AR, ERs, MR, and TRs. Both BGF and BPF exhibited similarly weak binding to ERs and moderate binding affinities to TRs. However, BGF demonstrated reduced binding affinities to GR and MR in comparison with BPF.

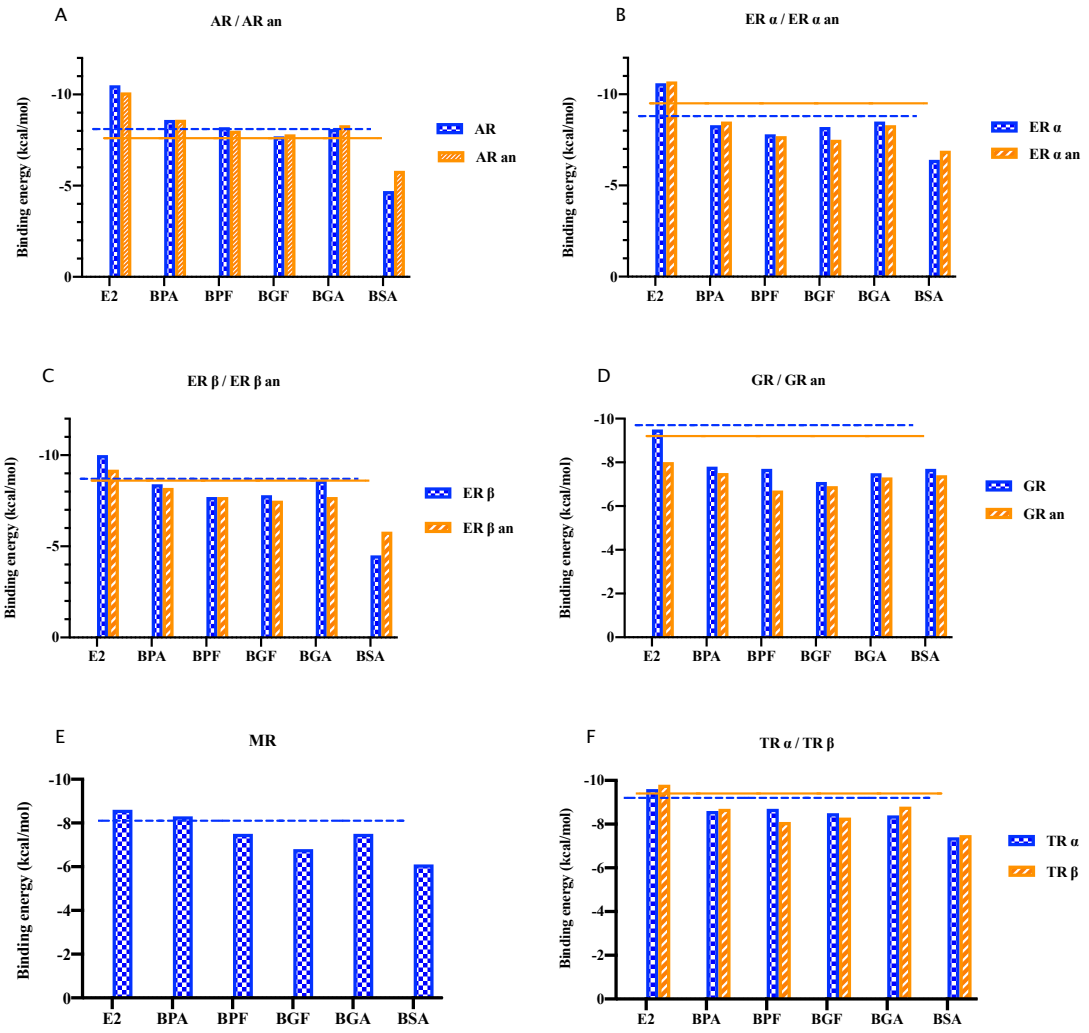

Figure 7.2: Binding energies (kcal/mol) of E2, BPA, BPF, BGF, BGA, and BSA to (A) AR and AR an, (B) ER α and ER α an, (C) ER β and ER β an, (D) GR and GR an, (E) MR, and (F) TR α and TR β.

The dashed line/solid line at each figure represents the threshold value of the strong binding for the respective receptor. The threshold values were generated by the docking tool that separated the strong-binding group and moderate-binding group.

The predicted LD_{50} values (for rats), developmental toxicity, and mutagenicity for the test compounds from T.E.S.T. are shown in Table A3. All the test compounds were classified as non-mutagenic developmental toxicants with values higher than the threshold of 0.5. For the acute toxicity to rats, the test chemicals had predicted LD_{50} values between 500 and 5000 mg/kg; thus, they were all classified in Category III (slightly toxic) on the basis of the EPA's 4-category hazard classification (Gadaleta et al., 2019). Category I ($LD_{50} \leq 50$ mg/kg) indicates the highest toxicity category, and Category IV ($LD_{50} > 5000$ mg/kg) indicates a safe chemical.

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

The potential EA of BPA, BPF, and three lignin-derivable monomers was investigated using the MCF-7 cell proliferation assay at concentrations ranging from 10^{-12} M to 10^{-6} M (1 pM to 1 μM), which covers the exposure level of BPA in different countries (Forde et al., 2022; Li et al., 2023). In the current study, we applied %RME2 of VC + 3 SD (18%) as a cut-off value for detectable EA (shown in Figure 7.3, dashed line) (Yang et al., 2014). Results showed that E2 had detectable EA at all test concentrations (from 10^{-12} to 10^{-6} M) with the maximum EA value of 95.4% ± 11% at 10^{-9} M. BPA had detectable EA at five test concentrations from 10^{-10} M to 10^{-6} M with the highest EA value at 49.5% ± 18% (10^{-6} M). BGA had detectable EA at three out of seven concentrations (10^{-9}, 10^{-8}, and 10^{-6} M) with a higher maximum EA value of 47% ± 12% (at 10^{-8} M). Notably, BSA had undetectable EA (< 18%) at seven

test concentrations. Next, BPF had the highest EA value of 42.6% ± 21% at 10^{-6} M, and it showed detectable EA from 10^{-8} M to 10^{-6} M. On the other hand, BGF showed detectable EA from 10^{-11} M to 10^{-9} M with a maximum EA of 28% ± 28% at 10^{-9} M. Compared with BPA, BGF had a significantly lower EA at 10^{-7} M ($p < 0.05$).

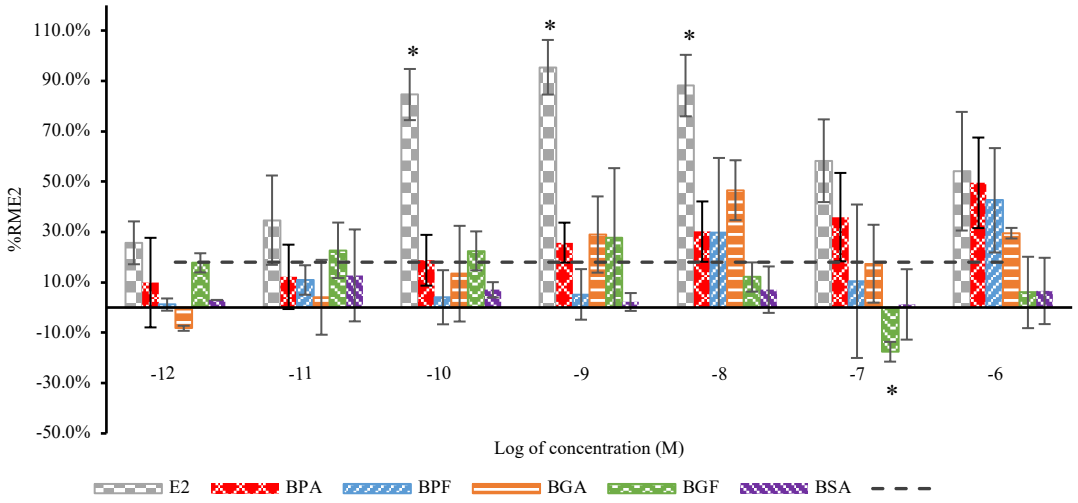

Figure 7.3: EA of E2, BPA, BPF, BGA, BGF, and BSA was quantified using the MCF-7 cell proliferation assay.

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

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

The hepatic mRNA expression levels of two estrogen-responsive genes (ApoII and VtgII) were assessed in the chicken embryos after exposure to E2, BPA, BPF, BGF, BGA, and BSA (at a non-toxic concentration of 0.1 mM). As shown in Figure 7.4, all treatments up-regulated ApoII gene expression in liver samples of day 16 embryos. E2, as the positive control, had a significantly higher fold change level (1198.51 ± 88.05) than other compounds. BPA had the highest fold change at 11.51 ± 3.36 among the other five treatments and was followed by BPF at 5.38 ± 2.45. Three lignin-derivable monomers had significantly lower expression levels (ranging from 1.84 to 4.41) of the ApoII gene in comparison to those of BPA and E2 ($p < 0.05$). On the other hand, except for the E2 group, the VtgII gene could not be consistently detected in other samples; thus, no fold change of gene expression was calculated (data not shown).

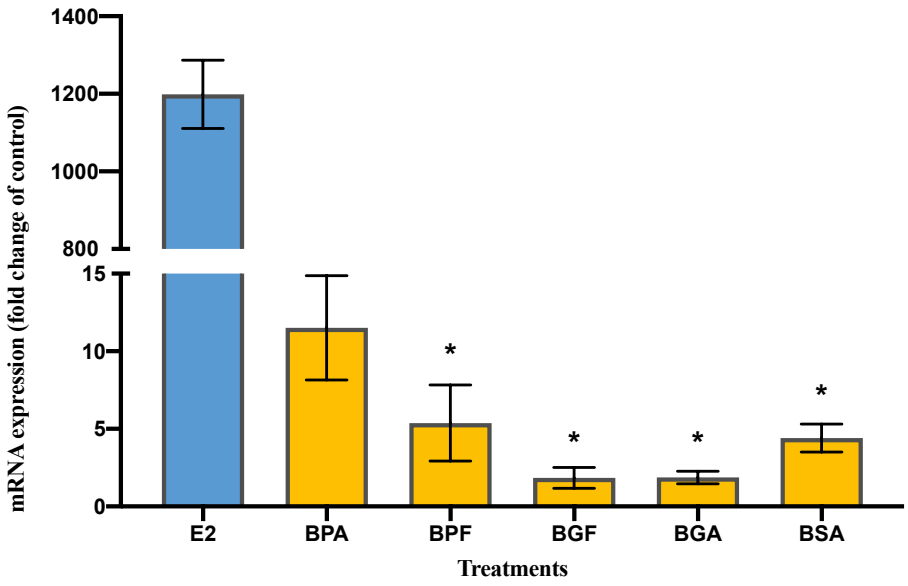

Figure 7.4: Effect of E2, BPA, BPF, and three lignin-derivable monomers on the mRNA expression of ApoII in the liver samples of day 16 female chicken embryo after injection at day 13 and day 15 at 0.1 mM.

Values are expressed as mean ± SD from three independent trials (n = 6), and significant changes are indicated relative to BPA (*$p < 0.05$; Dunnett's test).

7.4.4 Developmental toxicity assessment in chicken embryonic assay

7.4.4.1 Experiment 1: BPA treatment at five dosages

As shown in Table 7.1, highest exposure to BPA at 7600 µg/kg reduced the embryonic viability to 62.5% ($p < 0.05$), and the dosages at 76 µg/kg and 7.6 µg/kg both lowered the viability rate to 81.3%. The second highest dosage (760 µg/kg) and lowest dosage group (0.76 µg/kg) showed embryo viability of 94.8% and 87.5%, respectively. Additionally, deformed embryos (stunting) were found in three BPA

exposure groups at 0.76, 76, and 760 µg/kg. The REEW values were similar among the five BPA treatments and the VC from 0.40 ± 0.03 to 0.42 ± 0.04, except for the highest BPA dosage group, which had a slightly lower number of 0.37 ± 0.05. The largest LSI% number was detected as 2.99% ± 0.35% at the dosage of 7600 µg/kg and the second largest as 2.98% ± 0.30% at the dosage of 0.76 µg/kg, but with no significant difference from the VC (2.48% ± 0.28%) ($p > 0.05$). The liver weights showed a similar trend to the LSI% index after BPA exposure. However, due to the biological variance among the three trials, no significant difference was detected ($p > 0.05$).

Table 7.1: Effects of the BPA treatment at five exposure dosages on chicken embryonic viability, malformation status, REEW, LSI%, embryo weight, and liver weight of chicken embryos on day 18.

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

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

Our results showed that the VC had the lowest TBARS values of 79.03 ± 4.41 nmol/g and 94.03 ± 14.13 nmol/g for liver and brain samples, respectively (shown in Figure A1 A and B). BPA treatment at the highest dosage (7600 μg/kg) significantly increased the liver TBARS value to 101.75 ± 8.53 nmol/g ($p < 0.05$). Liver samples from the other four BPA dosages had increased TBARS values ranging from 88.69 ± 9.34 to 96.40 ± 9.90 nmol/g. However, only marginally elevated TBARS levels were found in the brain tissues after BPA exposure, ranging from 96.33 ± 22.17 to 105.30 ± 26.23 nmol/g without a statistically significant difference vs. the VC. Additionally, the VC had the highest CAT activity levels of 27.52 ± 6.54 and 0.31 ± 0.07 μmol/g for the liver and brain samples, respectively (shown in Figure A1C and D). In the liver samples, BPA treatment at five dosages decreased CAT activity values, ranging from 20.61 ± 2.38 to 22.96 ± 9.68 μmol/g ($p > 0.05$). A similar pattern of decreased CAT activity also was detected for brain tissues when exposed to BPA, ranging from 0.25 ± 0.08 to 0.29 ± 0.08 μmol/g, without significant change in comparison to the VC.

7.4.4.2 BPF, BGF, BGA, and BSA treatment at two dosages

Following the finding of BPA from Experiment 1, environmentally relevant exposure levels at injection concentrations 0.01 and 0.1 mM were applied in Experiment 2 to evaluate the developmental toxicity of three lignin-derivable monomers. E2 had a low viability rate of 70.6% (for 9.1 μg/kg) and 45.5% (91 μg/kg dosage) ($p < 0.05$). BGA and BSA showed viability rates of 75% and 87.5% at two test dosages that were comparable to that of BPA at 81.3%. On the other hand, BPF and BGF had the same viability rates of 100% (for low dosage) and 87.5% (for high dosage). Additionally, deformed embryos (*i.e.,* stunting and exposed brain) were detected in two E2 treatments at 11.8% and 9.1% for low and high dosages, respectively. The high-dosage BPA and the low-dosage BSA group also had a malformation rate of 12.5%. As shown in Table 7.2, BSA at the low dosage (11.6 μg/kg) had the lowest REEW value of 0.34 ± 0.03, which may be attributed to the stunted embryo detected in this group. The other groups had similar values ranging from 0.36 to 0.41. BPA, BPF, and three lignin-derivable monomers resulted in slightly higher LSI% values (2.56% - 2.77%) in comparison to the VC group of 2.31% ± 0.18% but with no statistical differences ($p > 0.05$).

Table 7.2: Effects of E2, BPA, BPF, BGA, BGF, and BSA treatments (at two injection concentrations) on chicken embryonic viability, malformation status, REEW, LSI (%), embryo weight, and liver weight of chicken embryos on day 18.

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

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

As shown in Figure A2, the VC had a TBARS value of 69.93 ± 8.60 nmol/g. E2 at the high dosage showed a significantly increased TBARS value of 99.20 ± 7.43 nmol/g vs. the VC ($p < 0.05$). At the low dosage treatment, E2 had TBARS level of 92.55 ± 3.39 nmol/g. BPA exposure raised the TBARS values to 90.25 ± 8.07 and 87.10 ± 14.36 for the low and high injection concentrations, respectively, but without a significant difference vs. the VC group. Additionally, at 0.01 mM BPF had a significantly decreased TBARS value vs. the BPA treatment ($p < 0.05$), whereas no statistically significant variations were found for other treatment groups vs the BPA treatment.

7.5 Discussion

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

We first applied molecular docking to three lignin-derivable monomers to predict the binding affinities of 14 nuclear receptors associated with the endocrine system. Results (Section 3.1) showed that BSA (with four methoxy groups *i.e.,* two methoxy groups per aromatic ring) had weaker binding affinities to the majority of receptors vs. BPA (with zero methoxy groups). However, BPA and BGA with two methoxy groups (*i.e.,* one methoxy group per aromatic ring) exhibited comparable binding affinities for most receptors (*e.g.*, ERs, GR, TRs). As one critical pathway of EDCs, the EA of test compounds was further investigated experimentally via the MCF-7 cell proliferation assay and fetal chicken hepatic mRNA expression of the

estrogen-responsive genes. Although the EA of BPA and bisphenol analogues (*e.g.,* BPF, BPAF, BPS) have been widely studied in the MCF-7 cell model (Rivas et al., 2002), two-hybrid yeast bioassays (Lei et al., 2017), and zebrafish-specific assays (Le Fol et al., 2017), much is unknown regarding the EA of lignin-derivable monomers.

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

affinities to ERs and undetectable EA in MCF-7 cells. Therefore, the two methoxy groups per aromatic ring provide sufficient steric restrictions around the phenolic hydroxyl group to reduce the interaction with binding sites. Together, the number of methoxy groups plays a critical role in reducing EA of dimethyl-substituted bridging bisphenols.

Additionally, we used a chicken embryo model targeting estrogenic-responsive genes to better understand the EA of lignin-derivable monomers. As ovipara, the chicken liver is a critical target organ for the steroid hormone estrogen, because it is the site of most of the yolk precursor protein synthesis (Li et al., 2014). Notably, the injection and sample collection time play important roles in the gene expression analysis of these two estrogen-responsive genes (VtgII and ApoII). The capacity of these two genes respond to estradiol fluctuation during the course of chicken embryo development; it peaked in the mid-incubation time and declined in the late fetal development stage (> embryonic day 17) (Evans et al., 1988). Thus, we chose the injection day at the mid-incubation time (day 13 - 15) and collected the sample before the target gene expression starts to decline, which is day 16. Among two estrogen-dependent genes (VtgII and ApoII) assessed, only the ApoII gene was successfully detected in all test groups, which agreed with literature reports that the induction of ApoII mRNA was a more sensitive endpoint than VtgII (Lorenzen et al., 2003). Therefore, only the ApoII gene was subjected to gene expression analysis in this study. Our results (see Figure 4) showed that the E2 significantly stimulated ApoII

expression with a roughly 1200-fold change vs. the control, which is consistent with an earlier study that E2 upregulated mRNA expression of ApoII in a dose-dependent pattern at 0.1 - 100 μg E2/egg (Li et al., 2014). Among the test substances, BPA exhibited the greatest effect on ApoII expression, with a fold change of 11.51 in comparison to the control. These results are in agreement with a study by Ma et al., in which BPA treatment increased ApoII mRNA levels in chicken embryonic hepatocytes in a concentration-dependent pattern (at 1 to 10 μM) (Ma et al., 2015). However, three lignin-derivable compounds (BGA, BSA, and BGF) had significantly lower fold change values (at 1.84 to 5.38) of ApoII expression than BPA ($p < 0.05$), indicating a lack of estrogenic response in the chicken fetal liver. Interestingly, BGA showed a lower EA than BPA in the gene expression assay, but not in the molecular docking and MCF-7 cell proliferation assay, which might be caused by different test systems and the mechanisms involved. The bisguaiacols/bissyringols have at least one methoxy group ortho to the hydroxyl group on each aromatic ring that may increase steric hindrance around phenolic hydroxyls and thus reduce interactions with binding pockets on the estrogen receptors that significantly lower the estrogenic-responsive gene expression level in comparison to BPA.

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

In addition to the EA, developmental toxicity is another important toxicity endpoint related to EDCs. The developmental toxicity of BPA and BPF has been

reported in various animal models, including rats (Lee et al., 2022), zebrafish (Gao et al., 2022), and chicken embryos (Mentor et al., 2020). To investigate the developmental toxicity of lignin-derivable monomers, we first applied T.E.S.T. to predict their acute toxicity and developmental toxicity (data shown in Table A3). Of note, even though the lignin-derivable monomers had varied oral rat LD_{50} values (~739 – 3196 mg/kg), they were assigned to Category III ($500 < \text{oral rat } LD_{50} \leq 5000$ mg/kg; slightly toxic) according to the EPA's 4-category hazard classification (Gadaleta et al., 2019). Although the QSARs-based simulations have been widely applied as valuable screening tools, it was still challenging to predict the developmental toxicity thoroughly due to the lack of dose response and its complex nature as several organs and hormones are involved (Hulzebos et al., 2001).

Therefore, an *in vivo* chicken embryonic assay was applied to further investigate the developmental toxicity of BPA and its potential alternatives. The chicken embryonic model has been recently recognized as a promising alternative model to traditional rodent animals for toxicological research (Ghimire et al., 2022). We first studied the developmental toxicity of BPA at five dosages ranging from 0.76 to 7600 μg/kg (at injection concentrations 0.001 to 10 mM) to cover the possible exposure level and explore the dose-response pattern. The results (shown in Table 1) revealed that BPA decreased the viability of chicken embryos with a non-monotonic dose response (NMDR). The highest mortality rate was detected at the highest dosage group (7600 BPA μg/kg egg) followed by two middle dosage groups (7.6 and 76 BPA μg/kg), whereas the second highest dosage treatment (760 BPA μg/kg) had the lowest

death rate. It has been widely reported that NMDR was involved in various BPA toxicities, such as hormone-sensitive endpoints and animal behavior studies (Yadav et al., 2022). However, the relevant mechanisms attributed to these NMDR relationships are still not fully understood, especially for *in vivo* modes (Lagarde et al., 2015).

To uncover the potential mechanism of oxidative stress on toxicity, we monitored two crucial biomarkers for the antioxidant system in chicken embryos: TBARS and CAT activity. TBARS serves as an indicator of oxidative damage in tissue samples, reflecting the lipid peroxidation levels (Kourouma et al., 2015). CAT has been identified as the main hydrogen peroxide scavenger, and is one of the crucial enzymes in the endogenous antioxidant defense system (Haider et al., 2021). Our results (Figure A1) showed that the highest dosage of BPA exposure significantly increased TBARS level in liver samples ($p < 0.05$), whereas for the brain samples, only marginally increased TBARS levels were detected after BPA exposure ($p > 0.05$). Additionally, a declining trend in CAT enzyme activity was discovered in liver samples after BPA treatments, but no significant difference was found due to the large biological variability. An impaired antioxidant enzyme system and increased lipid peroxidation after BPA exposure have been reported in rat and mouse models (Meng et al., 2019; Kourouma et al., 2015). For example, Kourouma et al. reported that BPA exposure significantly elevated malondialdehyde (MDA) levels and lowered CAT activity in the livers of rats in a dose-dependent manner. The difference in the previous study is that they used a considerably larger exposure dosage range (at 2 - 50 mg/kg) and a longer period (30 days) in comparison to our study, which may have caused a

noticeable increase in oxidative damage. Findings from CAT and TBARS assays indicated that the TBARS assay was a more sensitive assay for oxidative stress in response to low doses of BPA exposure at 0.76-7600 μg/kg.

Following the finding from BPA treatments, two intermediate and environmentally-relevant injection concentrations, 0.01 and 0.1 mM, were selected to determine the developmental toxicity of lignin-derivable monomers (Dekant and Völkel, 2008). In this study, E2 was used as a positive control. The results (shown in Table 2) revealed that E2 exposure at dosages of 9.1 (from 0.01mM injection) and 91 μg/kg led to significantly lower viability rates of chicken embryos, with rates of 70.6% and 45.5%, respectively, in contrast to the VC group. Except for the E2 groups, there was no significant difference in viability rates between other treatments and VC. BPF and BGF showed slightly higher viabilities (high dose: 87.5%; low dose: 100%) than BPA, whereas BGA and BSA had a similar level of viability as BPA. These results also agreed with the T.E.S.T. prediction that BPF and BGF had lower developmental values than BPA and BGA. This phenomenon may be because the dimethyl substituents in bisphenols increased their developmental toxicity. Previous studies also showed that BPF had lower developmental toxicity than BPA in the zebrafish embryo model regarding half-lethal concentrations and other developmental effects (Mu et al., 2018; Gao et al., 2022). More studies in other animals are still warranted to further understand the developmental toxicities.

On the basis of the results of Experiment 1, the liver was selected as the target organ for TBARS measurement, because it had a higher sensitivity than the brain

samples. A significantly increased TBARS value ($p < 0.05$) was only detected in the higher dosage of the E2 group in comparison to the VC (shown in Figure A2). In the past decade, the chicken embryo model has already been used for toxicity evaluation of BPA. BPA treatment at 200 mg/L (on embryonic day 4) significantly increased the MDA value and reduced the glutathione level in the chicken embryo brain, impairing the antioxidant defense system. A recent study reported that BPA exposure at 0.228 and 2.28 mg/egg damaged neural tube development in a chicken embryo at an early stage (28 to 76 h of incubation) (Atay et al., 2020). Additionally, it has been reported that BPA at 48 mg/kg induced an estrogen-like effect in the chicken embryo by disrupting reproductive organ development (Mentor et al., 2020). However, all these studies applied a higher dosage range, more than 1000-fold higher than the possible human exposure of BPA. In our study, we applied two exposure dosages of BPF and three lignin-derivable monomers from 6.7 to 116 μg/kg, which were much lower than the previous dose range of studies on BPA and were much closer to the average human intake, which is at 0.2 – 0.5 μg/kg body weight and can be up to 513.73 μg/person (Dekant and Völkel, 2008; Wang et al., 2020). Our chicken embryonic assay suggested that three lignin-derivable monomers (BGF, BGA, and BSA), as well as BPA and BPF, did not induce a significantly higher mortality rate nor elevated TBARS levels at the test dosages. Oxidative stress is normally considered as a major toxicity mechanism, so low TBARS levels might be one potential underlying mechanism for the low developmental toxicity of these compounds.

7.6 Conclusion

Herein, we evaluated the EA and developmental toxicity of dimethyl-substituted bridging carbon-based lignin-derivable bisphenols (BGA and BSA) for the first time *via* a multitiered method to advance efforts to replace environmentally harmful BPA. Notably, this work reinforces the importance of methoxy groups on lignin-derivable bisphenols in the reduction of toxicity concerns associated with bisphenols. Importantly, BSA with four methoxy substituents displayed weaker binding affinities to ERs and undetectable EA in the MCF-7 cell proliferation assay at 10^{-12} M to 10^{-6} M vs. BPA with zero methoxy groups. BPA exhibited detectable EA at 10^{-10} M to 10^{-6} M, whereas BGA showed detectable EA at only three out of seven test concentrations. Additionally, all the lignin-derivable compounds showed a lack of estrogenic response in the chicken fetal liver. Furthermore, our findings indicated that no clear adverse effects on chicken embryo development were detected after exposure to the three lignin-derivable monomers at an environmentally relevant exposure range. Together, the lignin-derivable monomers, especially BSA, reported in this study are potentially safer alternatives to BPA and other commercial bisphenols.

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Chapter 8

IN SILICO, IN VITRO, AND *IN VIVO* METABOLISM STUDY OF THREE LIGNIN-DERIVABLE MONOMERS AND BISPHENOL A

8.1 Abstract

With the increased toxicity concerns of bisphenol A (BPA) and commercial bisphenol analogs, lignin-derived bisphenols, bisguaiacols/bissyringols, have been proposed as promising and possibly safer alternatives to BPA. In this study, the *in vitro* metabolism of three lignin-derivable compounds, namely bisguaiacol F (BGF), bisguaiacol A (BGA), and bissyringol A (BSA), were investigated using liquid chromatography-mass spectrometry. As the first step, an *in silico* metabolite prediction tool: BioTransformer, based on machine learning approaches, was applied to predict the metabolite structures of these lignin-derivable compounds in humans. Additional analyses using high-performance liquid chromatography-fluorescent detection explored the distribution patterns of BPA in the major tissues of chicken embryos. In agreement with the predicted data, the experimental findings demonstrated that the three lignin-derivable compounds had identical *in vitro* metabolic pathways. These pathways resulted in glucuronide and sulfate conjugates from phase II metabolism, as well as a dealkylated metabolite from the phase I metabolic reaction. Additionally, the chicken embryo is a suitable model for metabolism studies.

8.2 Introduction

Bisphenol A (BPA) is a widely used synthetic phenolic compounds and has been used for the manufacturing of an array of polymeric systems. BPA-containing plastics have found applications in diverse fields, such as food packaging, pharmaceuticals, dental materials, flame retardants, and other products (Mikołajewska, Stragierowicz, and Gromadzińska 2015). The pervasive presence of BPA in human daily life has raised concerns about its potential impact on human health. Extensive research has explored the toxicity of BPA, linking it to endocrine disruption, reproductive and developmental toxicity, and genotoxicity (Vom Saal et al. 2012; Rubin 2011; Møller 2022). Due to these negative health impacts, numerous countries have imposed restrictions on the use of BPA in food contact materials, particularly in products designed for infants and children (Erler and Novak 2010; Usman and Ahmad 2019; Usman and Ahmad 2019).

In the past two decades, several bisphenol analogs have been produced as BPA alternatives and quickly used in different products. However, the toxicity concerns of these commercial bisphenol analogs have also been revealed (Lei et al. 2017; Harnett et al. 2021; Yin et al. 2019). Recently, lignocellulosic biomass, a sustainable resource, has gained great attention (Nicastro, Kloxin, and Epps, III 2018) and the lignin-derivable bisphenols are suggested as safer alternatives to commercial bisphenols. For example, Bisguaiacol F (BGF) has been reported as one of promising BPA alternatives with comparable thermomechanical properties to BPA while lowering concern on estrogenic activity (Peng et al. 2018).

The metabolism of chemicals plays an important role in chemical toxicology, since metabolites might have a very different toxicity profile than the parent compounds (Zheng et al. 2020). Ramirez et al. systematically reviewed the metabolic pathway of BPA and bisphenol analogs (BPS, BPF, BPAF, and BPB). BPA-monoglucuronide is one predominant metabolite formed in vivo by hepatic phase II enzyme (UGTs). This major metabolite isolated from adult female F-344 and CD rats was reported as having no estrogenic activity (Snyder et al., 2000). BPA-sulfate is another major phase II metabolism product in vivo by sulfotransferases (SULTs) (Skledar and Mašič 2016). However, phase I hydroxylated metabolites of BPA catalyzed by microsomal cytochrome P450 (CYP450), were shown with higher toxicity concerns (Nakamura et al. 2011).

Understanding the metabolic pathway of these lignin-derivable compounds is important before applying them in manufacturing. In this study, we investigated the in vitro metabolic pathways of bisguaiacol F (BGF) and two dimethyl-substituted bridging carbon-based lignin-derivable bisphenols (bisguaiacol A [BGA] and bissyringol A [BSA]). The aims of this study were first to explore the in vitro metabolic pathway of three lignin-derivable monomers (BGF, BGA, and BSA) and BPA using human liver microsomes (HLMs) to produce Phase I and Phase II metabolites. Second, using a chicken embryo to investigate a tissue distribution pattern of BPA. The potential phase I and phase II metabolites were synthesized and characterized by liquid chromatography coupled to tandem mass spectrometry (LC-MS).

8.3 Materials and methods

8.3.1 Chemicals

BGA (> 99%), BSA (> 99%), and BGF (> 99%) were synthesized according to published literature (Epps, III et al. 2022; Nicastro, Kloxin, and Epps, III 2018). Magnesium chloride ($MgCl_2$), uridine 5′-diphosphoglucuronic acid ammonium salt (UDPGA), β-Nicotinamide adenine dinucleotide phosphate (NADPH), phosphate buffer solution, perchloric acid, adenosine-3′ -phosphate 5′ -phosphosulfate (PAPS, > 60%), and alamethicin were obtained from Sigma-Aldrich (St. Louis, MO). Human liver microsomes (HLMs; 20 donor pool) was purchased from Corning (Corning, NY).

8.3.2 *In silico* metabolite structure prediction

BioTransformer 3.0 was applied to predict the potential metabolites structures of BGF, BGA, and BSA (https://biotransformer.ca). Three major types of Metabolic Transformation: phase I reactions (cytochrome P450), phase II reactions, and human gut microbial reactions were selected. SMILES string of test compounds that generated by ChemDraw were put into the text box and the default number (1) of Reaction Iterations to Calculate was choose.

8.3.3 *In vitro* metabolism assay

8.3.3.1 Phase 1 metabolic reaction

Test chemicals (at 5 mM, dissolved in methanol) were incubated with HLMs (1 mg/mL) in a reaction mixture (at 100 μL) containing 50 mM phosphate buffer (pH 7.4) and 5 mM $MgCl_2$. The reaction mixture, with 1% methanol, was pre-incubated at 37 °C for 5 min and was initiated by the addition of NADPH at a final concentration of 1 mM. The mixture was incubated at 37 °C for 1 h and the reaction was terminated by addition of 25 μL ice-cold acetonitrile. The tubes were transferred to -20 °C for 24 h and centrifuged at 16,000 ×g for 10 min at 4 °C. The supernatants were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses following the procedure described by Skledar et al. (2016).

8.3.3.2 Glucuronidation assay

The glucuronidation assay was performed as describe in Skledar et al. (2019). Briefly, test chemicals (at 5 mM) were incubated with HLM (0.2 mg/mL), 50 mM phosphate buffer, pH 7.4, 10 mM $MgCl_2$, 5 mg alamethicin, and 2% DMSO in a final volume of 100 μL. The reaction mixture was kept on ice for 30 min and pre-incubated at 37 °C for 5 min. The reaction was initiated by addition of UDPGA at a final concentration of 5 mM and incubated at 37 °C for 1 h. Ten μL 70% perchloric acid was added into the mixture to terminate the reaction and the tubes were kept on ice for 15 min. Then, the tubes were centrifuged at 16 000 ×g for 10 min and the supernatants were collected for LC-MS analysis.

8.3.3.3 Sulfation assay

The chemicals were incubated with HLM (1.5 mg/mL), 50 mM phosphate buffer, pH 7.4, 5 mM $MgCl_2$ at a final volume of 100 µL. After pre-incubated at 37 °C for 5 min, PAPS (at 50 µM) was added to initiate the reaction and incubated for at 37 °C for 1 h. After incubation the reaction was quenched by adding 25 µL ice-cold acetonitrile, and kept on ice for 15 min. The tubes were centrifugated at 16 000 ×g for 10 min and the supernatants were subjected for LC-MS analysis (Sonker et al. 2021).

8.3.3.4 LC-MS/MS analysis

LC-MS/MS analysis was performed on a Xevo TQ-XS coupled to an Acquity Premier UPLC (Waters Corporation, Milford MA). Chromatographic separation was achieved using an Acquity Premier BEH C18 1.7 µm, 2.1 x 50 mm column. Mobile phase A was water containing 0.1% formic acid and mobile phase B was acetonitrile containing 0.1% formic acid. The elution consisted of a linear gradient going from 5% B to 95% B in 4 min at a column temperature of 40 °C. The injection volume was 5 µL.

The MS parameters were set as follows: negative ionization mode; capillary voltage 2.50 kV, cone voltage 9 V, desolvation temperature 250 °C, desolvation gas flow 600 L/Hr, cone gas 150 L/hr, and nebulizer gas 7 bar. The analytes were measured in the multiple reaction monitoring mode (MRM). The ion transitions were detected along with their optimized collision energies (20 ~ 30 eV).

8.3.3.5 Mass spectrometry analysis

Fragmentation experiments were conducted using an UltiMate 3000 UHPLC with autosampler for direct injection coupled to an Orbitrap Q-Exactive with a heated electrospray ionization (HESI) source operated in negative mode for ESI MS/MS analysis. All analytes were first run in full scan mode to confirm ionization as the pseudomolecular ion. The MS/MS experiments used flow-injection through a union with 2 μL injection of 50 μM standards in methanol. The loading phase was 0.1% (v/v) ammonia solution (20-22% NH_3, Optima, Thermo Fisher) in 90% LC-MS grade methanol and 10% LC-MS grade water. Analysis was targeted selected ion monitoring, data-dependent MS/MS (t-SIM-ddMS2) with monitoring windows centered on the [M−H]- theoretical values and 1 m/z isolation windows for SIM and MS/MS. The following parameters were applied in the mass spectrometer: sheath gas flow (N_2), 45; auxiliary gas flow (N_2), 10; sweep gas flow (N_2), 2; spray voltage, -3.5 kV; capillary temperature, 275 °C; S-lens RF level, 50; auxiliary gas heater, 400. Collision was higher-energy collisional dissociation (HCD), and for BGA, BSA, and BGF was set at normalized collision energy (NCE) of 30, while 60 NCE was chosen for BPA. These were selected to optimize fragmentation but with continued observation of the precursor.

8.3.4 In vivo metabolism study using chicken embryo model

8.3.4.1 Egg treatment and sample extraction

BPA at 10 mM was injected at 0.2 mL into egg on embryonic day 16. The blood and tissues (liver and brain) were collected after 1, 2, 6, and 24 hours. The liver/brain samples of 0.15 g were weight and homogenized with 300 µL ammonium acetate buffer (0.01 M, pH 4.5) at room temperature. Volumes of 1.2 mL of methanol and 15 µL of perchloric acid (4 M) were added to the homogenate. The sample was mixed, sonicated (10 min), and centrifuged (10 min, 12,000 rpm). The supernatant (1 mL) was transferred to a glass tube and combined with 2 mL ammonium acetate buffer (0.01 M, pH 4.5).

The C18 SPE cartridge (200 mg, Hypersep, USA) was conditioned with 2 ml of methanol and equilibrated with 1 ml of water and 1 ml of 0.01 M ammonium acetate buffer (pH 4.5) prior to use. The sample solution was loaded onto the cartridge, washed with 2 mL of water, and eluted with 3 mL of methanol. The eluting solution was evaporated to dryness under N_2 and reconstituted in 100 µL of methanol. The obtained samples were analyzed by HPLC.

An aliquot (0.2 mL) of serum was extracted by adding 1.2 mL of ethyl acetate. The mixtures were shaking for 10 min and centrifuged for 10 min at 5000 rpm. The samples were extracted twice, and the organic layers were combined and transferred to a clean glass. Then the extracts were evaporated to dryness under N_2 and reconstituted in 100 µL of methanol. The obtained samples were analyzed by HPLC.

8.3.4.2 Enzyme treatment

For analysis of the BPA metabolites, 0.15 g of liver or brain samples were homogenized with 0.3 mL of ammonium acetate buffer (1 M, pH 5.0), then 10 µL of ß-glucuronidase (1000 unit) was added and vortexed for 1 min. The sample was incubated at 37 °C for 16 h. For serum samples, an aliquot of 0.2 mL sample was added into 0.4 mL of ammonium acetate buffer (1 M, pH 5.0) with 3 µL of ß-glucuronidase (300 units). The sample was vortexed for 1 min and incubated at 37 °C for 16 h. The enzymatic reactions were terminated by placing on ice and total BPA was exacted as described above.

8.3.4.3 HPLC analysis

HPLC (Shimadzu) equipped with fluorescent and PDA detectors was used for quantification of BPA. Chromatographic separation was achieved using an XBridge C18 3.5 µm, 4.6 x 150 mm column. Mobile phase A was water and mobile phase B was acetonitrile. The system was run in a linear gradient: 0–2 min A60%, B40%, 2–12 min A30%, B70%, 12-14 min A 5%, B-95%, 14-17 min 5%, B95%, 17-18 min A60%, B40%, and 18-22 min A60%, B40%. The column temperature was 35 °C and the flow rate of the mobile phase was 0.75 mL/min. The injection volume was 20 µL. The fluorescence detector was set at an excitation wavelength of 230 nm and an emission wavelength of 315 nm.

8.4 Results and discussion

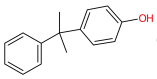
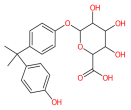
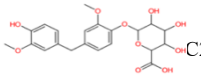
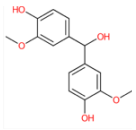
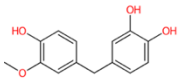
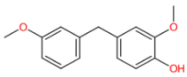
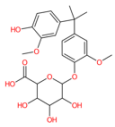
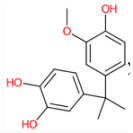
8.4.1 Metabolism prediction by Biotransformer

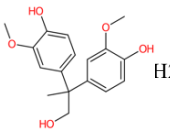
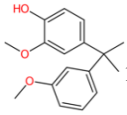
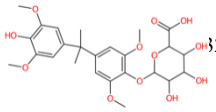
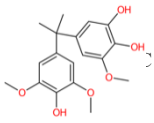
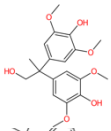
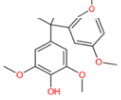
Recently, the application of *in silico* metabolism predictions in research has been increasing (Kazmi et al. 2019). It is considered a cost- and time-saving means to identify the structures of *in vitro* metabolites. In a recent study comparing simulated computational results to experimental data on several bisphenols, BioTransformer exhibited the best performance among three prediction programs (Chemical transformation simulator, BioTransformer, and Meteor Nexus) (Bruks 2020).

In the current study, BioTransformer, which utilizes machine learning approaches with a rule-based system, was applied as the first step to predict the possible metabolite structures of three lignin-derivable compounds in humans (Wishart et al. 2022). The predicted metabolites of BPA, BGF, BGA, and BSA via phase 1 transformation, phase 2 transformation, and human gut microbial transformation are listed in Table 8.1. The results showed that the three lignin-derivable compounds had high similarity in their metabolite structures, with the same reaction types for each metabolic transformation category. The glucuronide conjugates were the major phase 2 metabolites for three lignin-derivable compounds. Two predicted reaction types for the phase 1 transformation of the three compounds were hydroxylation and dealkylation. However, there were no data available for BPA regarding phase 1 transformation and phase 2 transformation prediction. For the human gut microbial transformation, BPA exhibited the same predicted reaction type,

dehydroxylation, as the other three compounds, along with one additional glucuronidation reaction.

Table 8.1: Metabolism prediction results of BGF, BGA, and BSA from Biotransformer 3.0.

Compound	Metabolic transformation type	Predicted result	Chemical formula	Major isotope mass (Da)	Reaction type	Reaction info
BPA	Human gut Microbial transformation		C15H16O	212.1201	4'-Dehydroxylation of substituted benzene	Enzyme: Unspecified bacterial dehydroxylase BioSystem: GUTMICRO
			C21H24O8	404.1471	Aromatic OH-glucuronidation	Enzyme: Bacterial UDP-glucuronosyltransferase BioSystem: GUTMICRO
BGF	Phase 2 transformation		C21H24O10	436.1369	Aromatic OH-glucuronidation	Enzyme: UDP-glucuronosyltransferase BioSystem: HUMAN
	Phase 1 transformation		C15H16O5	276.0997	Hydroxylation of non-terminal aliphatic carbon adjacent to aromatic ring	Enzyme: Cytochrome P450 1A2 BioSystem: HUMAN
			C14H14O4	246.0892	Dealkylation	Enzyme: Cytochrome P450 1A2 BioSystem: HUMAN
	Human gut Microbial transformation		C15H16O3	244.1099	4'-Dehydroxylation of substituted benzene	Enzyme: Unspecified bacterial dehydroxylase BioSystem: GUTMICRO
BGA	Phase 2 transformation		C23H28O10	464.1682	Aromatic OH-glucuronidation	Enzyme: UDP-glucuronosyltransferase BioSystem: HUMAN
	Phase 1 transformation		C16H18O4	274.1205	O-Dealkylation	Enzyme: Cytochrome P450 1A2 BioSystem: HUMAN

		 H20O5	304.131	Hydroxylation of terminal methyl	Enzyme: Cytochrome P450 1A2 BioSystem: HUMAN
	Human gut Microbial transformation	 17H20O3	272.1412	4'-Dehydroxylation of substituted benzene	Enzyme: Unspecified bacterial dehydroxylase BioSystem: GUTMICRO
BSA	Phase 2 transformation	 32O12	524.1893	Aromatic OH-glucuronidation	Enzyme: UDP-glucuronosyltransferase BioSystem: HUMAN
	Phase 1 transformation	 18H22O6	334.1416	O-Dealkylation	Enzyme: Cytochrome P450 1A2 BioSystem: HUMAN
		 C19H24O7	364.1522	Hydroxylation of terminal methyl	Enzyme: Cytochrome P450 1A2 BioSystem: HUMAN
	Human gut Microbial transformation	 C19H24O5	332.1623	4'-Dehydroxylation of substituted benzene	Enzyme: Unspecified bacterial dehydroxylase BioSystem: GUTMICRO

8.4.2 Metabolite results from *in vitro* metabolism assay

Full-scan LC-MS was performed first to check the possible metabolites of BPA and the three lignin-derivable compounds under three different incubation conditions. Four glucuronide conjugates, BPA glucuronide (BPA-G), BGF glucuronide (BGF-G), BSA glucuronide (BSA-G), and BGA glucuronide (BGA-G), were detected after the glucuronidation reaction of each test compound. Similarly, four coresponding sulfate conjugates, BPA sulfate (BPA-S), BGF sulfate (BGF-S), BSA sulfate (BSA-S), and BGA sulfate (BGA-S), of four test compounds were identified after the sulfation assay. For the identification of Phase I metabolites for BGF, BSA, and BGA, we searched for the two predicted structures from Phase I transformation by

the Biotransformer. We found that only the metabolites generated from the dealkylation reaction were detected for the three test compounds (indicated as M1 in Table 2). Additionally, we identified one phase 1 metabolite of BPA , full name (BPA+O), which has been reported in previous studies (Ousji, Ohlund, and Sleno 2020) as a major oxidative metabolite of BPA. After identifying the metabolites, MS/MS experiments were conducted to detect the two major fragment ions using different collision energies (as listed in Table 8.2).

The three lignin-derivable monomers and BPA formed $[M–H]^-$ ions in negative ion mode, and accurate m/z values were obtained for the product ions in MS/MS spectra (Table 8.3). The fragmentation pathway of BPA has been widely studied (Zhao et al. 2016; Ousji, Ohlund, and Sleno 2020). In agreement with those two publications, our results showed that the major fragment ions of BPA were observed at m/z 211.07541, 133.06382, and 93.03249. Interesting, the ESI-MS/MS spectra of the $[M–H]^-$ ions of the three lignin-derivable compounds showed product ions formed common product ion $[M\text{-}H\text{-}CH_3]^-$ and $[M\text{-}H\text{-}C_2H_6]^-$.

Table 8.2: Molecular weight, measured mass, two reaction monitoring transitions, retention time, and collision energies of test chemicals.

Compound	Molecular Formula	Measured Mass [m/z]	Transition (m/z)	Retention Time (min)	Collision Energies (eV)
BPA-G	$C_{21}H_{24}O_8$	403	403 > 174.86; 403 > 226.99	2.53	20

BPA-S	$C_{15}H_{16}O_5S$	307	307 > 172; 307 > 227	2.71	25
BPA+O	$C_{15}H_{16}O_3$	243	243 > 181.04; 243 > 224.97	3.16	25
BGF-G	$C_{21}H_{24}O_{10}$	435	435 > 258.98; 435 > 243.95	2.34	25
BGF-S	$C_{15}H_{16}O_7S$	339	339 > 244; 339 > 259	2.44	25
BGF M1	$C_{14}H_{14}O_4$	245	245 > 180.88; 245 > 228.99	2.52	25
BSA-G	$C_{25}H_{32}O_{12}$	523	523 > 332.04; 523 > 347.05	2.66	30
BSA-S	$C_{19}H_{24}O_9S$	427	427 > 332; 427 > 347	2.58	25
BSA M1	$C_{18}H_{22}O_6$	333	333 > 265; 333 > 285	3.92	25
BGA-G	$C_{23}H_{28}O_{10}$	463	463 > 272; 463 > 287.03	2.69	25
BGA-S	$C_{17}H_{20}O_7S$	367	367 > 272; 367 > 287	2.77	20
BGA M1	$C_{16}H_{18}O_4$	273	273 > 243; 273 > 258	3.25	25

Table 8.3: Elemental compositions and mass data of the precursor ions and their main product ions for BPA, BGF, BGA, and BSA.

Compound	Ion species	Elemental component	Measured mass (m/z)	Theoretical mass (m/z)	Relative error (ppm)
BPA	$[M-H]^-$	$C_{15}H_{15}O_2$	227.10697	227.10775	-3.44
	$[M-H-CH_4]^-$	$C_{14}H_{11}O_2$	211.07541	211.07645	-4.94
	$[M-H-C_6H_6O]^-$	C_9H_9O	133.06382	133.06589	-15.54
	$[M-H-C_9H_{10}O]^-$	C_6H_5O	93.03249	93.03459	-22.57
BGF	$[M-H]^-$	$C_{15}H_{15}O_4$	259.09781	259.09758	0.87
	$[M-H-CH_3]^-$	$C_{14}H_{12}O_4$	244.0738	244.07411	-1.27

	$[M-H-C_2H_6]^-$	$C_{13}H_9O_4$	229.05001	229.05063	-2.74
	$[M-H-C_{12}H_{12}O_1]^-$	$C_3H_3O_3$	87.00671	87.00877	-23.62
BGA	$[M-H]^-$	$C_{17}H_{19}O_4$	287.12924	287.12888	1.23
	$[M-H-CH_3]^-$	$C_{16}H_{16}O_4$	272.10571	272.10541	1.11
	$[M-H-C_2H_6]^-$	$C_{15}H_{13}O_4$	257.08201	257.08193	0.32
	$[M-H-C_3H_9]^-$	$C_{14}H_{10}O_4$	242.05793	242.05846	-2.19
	$[M-H-C_{14}H_{16}O_1]^-$	$C_3H_3O_3$	87.00672	87.00877	-23.53
BSA	$[M-H]^-$	$C_{19}H_{23}O_6$	347.15017	347.15001	0.44
	$[M-H-CH_3]^-$	$C_{18}H_{20}O_6$	332.12665	332.12654	0.35
	$[M-H-C_2H_6]^-$	$C_{17}H_{17}O_6$	317.10315	317.10306	0.28
	$[M-H-C_{12}H_{12}O_3]^-$	$C_7H_{11}O_3$	143.06948	143.07137	-13.18
	$[M-H-C_{16}H_{20}O_3]^-$	$C_3H_3O_3$	87.00672	87.00877	-23.53

8.4.3 Tissue distribution of BPA in a chicken embryo model

The average time-tissue concentration for free BPA and conjugated BPA after injection of 10 mM BPA (7.6 μg/g egg) are shown in Figure 1. As shown in Figure 1A, aglycone BPA was detected in both liver and brain tissues at 1-6 h after BPA injection at a comparable level (from 403.63 μg/mL to 39.01μg/mL for liver tissue; from 313.28 μg/mL to 33.88 μg/mL). Only a low level of BPA could be detected in both tissue samples after a 24-h treatment. Figure 1B shows that after β-glucuronidase treatment, the amount of hydrolyzed BPA detected in the liver was significantly higher than that found in the brain. The total hydrolyzed BPA found in the liver was over 10-

fold higher than in the brain after a 1-h treatment. In addition, the total level of BPA in liver samples rapidly dropped after a 2-h injection. These results suggest that the embryonic liver is the major organ for metabolizing BPA, and BPA is quickly metabolized to BPA-G in chicken embryos. Importantly, a small amount of BPA was detected in the liver and brain tissues of our control chicken embryos without BPA injection, with average concentrations of 8.8 ng/mL and 12.3 ng/mL in the liver and brain, respectively.

Metabolic reaction and pharmacokinetics patterns of BPA have been widely studied in different in vivo models (e.g., rats and rhesus monkeys) (Doerge, Twaddle, Vanlandingham, et al. 2010) (Doerge, Twaddle, Woodling, et al. 2010). In agreement with our results, Doerge et al. reported that BPA-G was the dominant BPA conjugate and emphasized the critical role of Phase II metabolism of BPA in the rat liver.

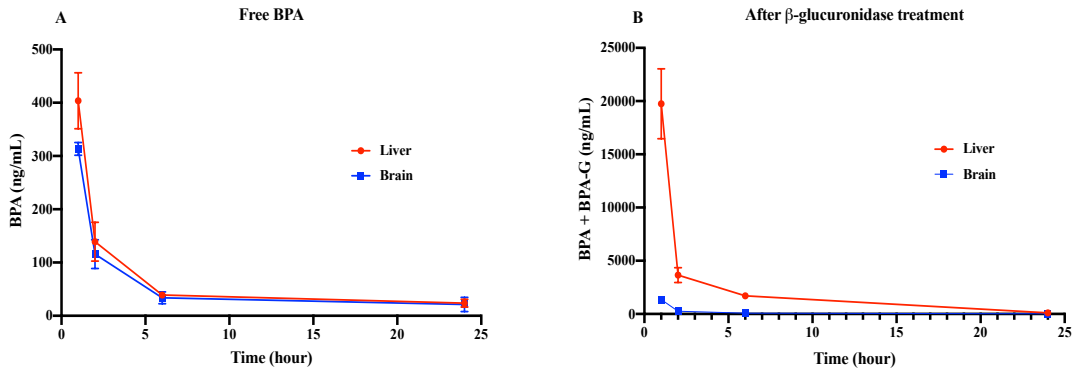


Figure 8.1: Tissue concentration-time profiles of (A) free BPA and (B) free BPA + BPA-G. Numbers were presented as mean ± standard deviation (SD) from two trials (n = 4).

8.5 Conclusion

This study investigated the *in vitro* metabolic pathway of three lignin-derived bisphenols in HLM. To aid in the identification of potential metabolite structures, BioTransformer was applied as the first step—an *in silico* prediction tool. Our results showed that these three tested lignin-derived bisphenols undergo the same metabolic reactions as BPA through phases I and II. Additionally, we explored the tissue distribution pattern of BPA and its major metabolite BPA-G using a chicken embryo model. We propose that the chicken embryo could be a suitable alternative model for metabolism studies.

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Chapter 9

SUMMARY AND FUTURE RESEARCH

The first part of studies in this project showed that natural phenolic compounds (such as monoterpenoid and flavonoids) raised health concerns regarding estrogenic activity (EA) and developmental toxicity during chicken embryogenesis varied depending on their structures. Additionally, lignin derivable monomers tested in this study demonstrated overall lower EA compared to Bisphenol A (BPA) among different test methods, while they showed comparable developmental toxicity to BPA via a chicken embryonic assay.

The future research would be conducted in three areas. First, aim to further standardize and improve the chicken embryo model for toxicology studies by utilizing well-established chemicals for testing. Connect exposure dosages to the mother's placental doses and explore other sensitive biomarkers of toxicity. Second, explore the toxicity profiles of the metabolites of lignin-derivable compounds. Even though these compounds have a similar in vitro metabolism pattern to BPA, it is still worthwhile to investigate the toxicity, especially the EA, of their major metabolites. Last, generate a database that correlates chemical structure with different toxicity activity. This

database would assist in avoiding the synthesis of compounds with undesirable toxicity during further chemical development.

SUPPLEMENTARY INFORMATION OF CHAPTER 7

Table A1: Primer sequences for β-actin, apolipoprotein II (ApoII), and vitellogenin II (VtgII) genes.

Gene name	Forward primer	Reverse primer	Probe	Accession number
β-actin	AAATTGTGCGTGCATCAAGGA	GAGGCAGCTGTGGCCATCT	TGCTACGTCGCACTGGATTTGGAGC	DQ207609
ApoII	CTGGGAGAGAGAAAGCAGGA	AATGCCCTGTATTGCACCAT	TACCAGTTCAGCCCTT-TACACCAAGAGACC	J00810
VtgII	CTGGGACTGCCGGGACTA	AGGGACAAACTCGTAAAACCATTC	CCGCCCAGTTAC-CAGCTCAGTAGAAACCT	M18060

Table A2: Binding energies (kcal/mol) of 17β-estradiol (E2), bisphenol A (BPA), bisphenol F (BPF), bisguaiacol F (BGF), bisguaiacol A (BGA), and bissyringol A (BSA) to 14 nuclear receptors.

Receptors	E2	BPA	BPF	BGF	BGA	BSA
AR	-10.5	-8.6	-8.2	-7.7	-8.1	-4.7
AR an	-10.1	-8.6	-8.0	-7.8	-8.3	-5.8
ER α	-10.6	-8.3	-7.8	-8.2	-8.5	-6.4
ER α an	-10.7	-8.5	-7.7	-7.5	-8.3	-6.9
ER β	-10.0	-8.4	-7.7	-7.8	-8.6	-4.5
ER β an	-9.2	-8.2	-7.7	-7.5	-7.7	-5.8
GR	-9.5	-7.8	-7.7	-7.1	-7.5	-7.7
GR an	-8.0	-7.5	-6.7	-6.9	-7.3	-7.4

LXR α	-10.4	-8.6	-8.2	-8.5	-8.7	-8.3
LXR β	-10.8	-8.1	-7.8	-8.1	-8.3	-8.3
MR	-8.6	-8.3	-7.5	-6.8	-7.5	-6.1
PPAR α	-8.0	-7.8	-7.5	-7.4	-7.1	-7.3
PPAR β	-8.2	-7.9	-7.2	-7.2	-7.5	-7.9
PPAR λ	-8.0	-7.5	-7.2	-6.8	-7.2	-7.7
PR	-2.7	-2.4	-2.6	-2.3	-2.4	-2.6
RXR	-8.8	-7.9	-8.1	-8.1	-8.0	-7.9
TR α	-9.6	-8.6	-8.7	-8.5	-8.4	-7.4
TR β	-9.8	-8.7	-8.1	-8.3	-8.8	-7.5

Table The results were sorted into four classes: red (very strong binding), orange (strong binding), yellow (moderate binding), and no highlight (weak binding). AR: androgen receptor, ER: estrogen receptor, GR: glucocorticoid receptor, LXR: liver X receptor, MR: mineralocorticoid receptor, PPAR: peroxisome proliferator-activated receptor, PR: progesterone receptor, RXR: retinoid X receptor, TR: thyroid receptor, an: antagonist conformation.

Table A3: Oral rat median lethal dose (LD_{50}) values, developmental toxicity, and mutagenicity values of E2, BPA, BPF, BGF, BGA, and BSA simulated by the Toxicity Estimation Software Tool.

Compound	Oral rat LD_{50}		Developmental toxicity		Mutagenicity	
	Log10 (mol/kg)	LD_{50} (mg/kg)	Value	Result	Value	Result
E2	2.36	1186	0.95	Developmental toxicant	0.23	Mutagenicity negative
BPA	1.85	3196	0.68	Developmental toxicant	0.09	Mutagenicity negative

BPF	1.85	2840	0.57	Developmental toxicant	0.26	Mutagenicity negative
BGF	2.03	2429	0.71	Developmental toxicant	0.26	Mutagenicity negative
BGA	2.59	738	1.07	Developmental toxicant	0.12	Mutagenicity negative
BSA	2.41	1347	0.71	Developmental toxicant	0.10	Mutagenicity negative

Note: Based on the simulated oral rat LD_{50} values (between 500 and 5000 mg/kg), all test compounds were placed in Category III (slightly toxic) according to the Environmental Protection Agency's 4-category hazard classification. Category I ($LD_{50} \leqslant 50$ mg/kg) indicates the highest toxicity category, and Category IV ($LD_{50} > 5000$ mg/kg) indicates a safe chemical.

Table A4: The half maximal effective concentration (EC_{50}) and the highest relative maximum %E2 (%RME2) of test compounds as determined from the MCF-7 cell proliferation assays.

Compound	E2	BPA	BPF	BGA	BGF	BSA
EC_{50} (M)[a]	1.29×10^{-11}	1.48×10^{-9}	3.58×10^{-9}	6.76×10^{-11}	1.11×10^{-8}	N/A
Max %RME2[b]	95.4%	49.5%	42.6%	47%	28%	13%

[a] EC_{50} of test compounds was calculated using GraphPad Prism. N/A means unavailable data because BSA had undetectable estrogenic activity at all test concentrations.

[b] Max %RME2 was the highest estrogenic activity value using a test range of 10^{-12} - 10^{-6} M as determined from MCF-7 cell proliferation assays.

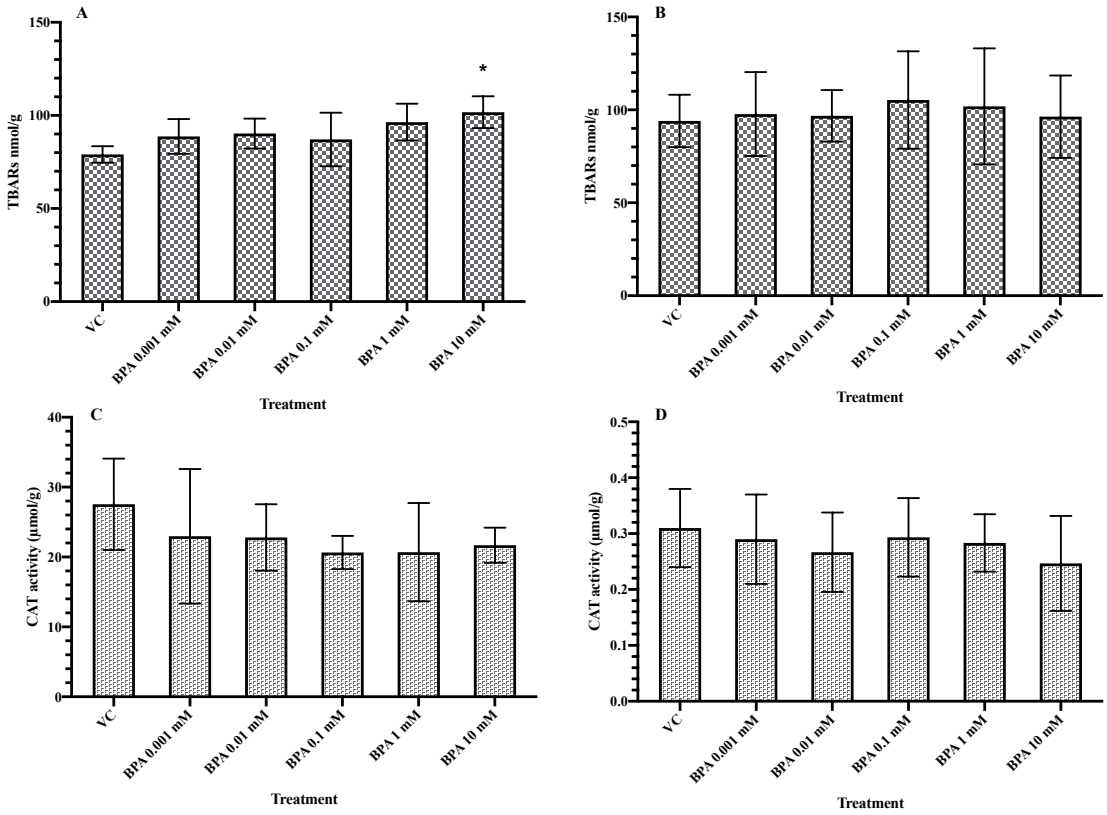

Figure A1: Effects of five BPA treatments on the thiobarbituric acid reactive substance (TBARS) value (A: liver samples, B: brain samples) and catalase (CAT) activity (C: liver samples, D: brain samples) of day 18 chicken embryos.

Values were expressed as mean ± standard deviation (SD) from three independent trials. Differences were evaluated using one-way analysis of variance followed by Dunnett's test compared with the vehicle control (VC) group.

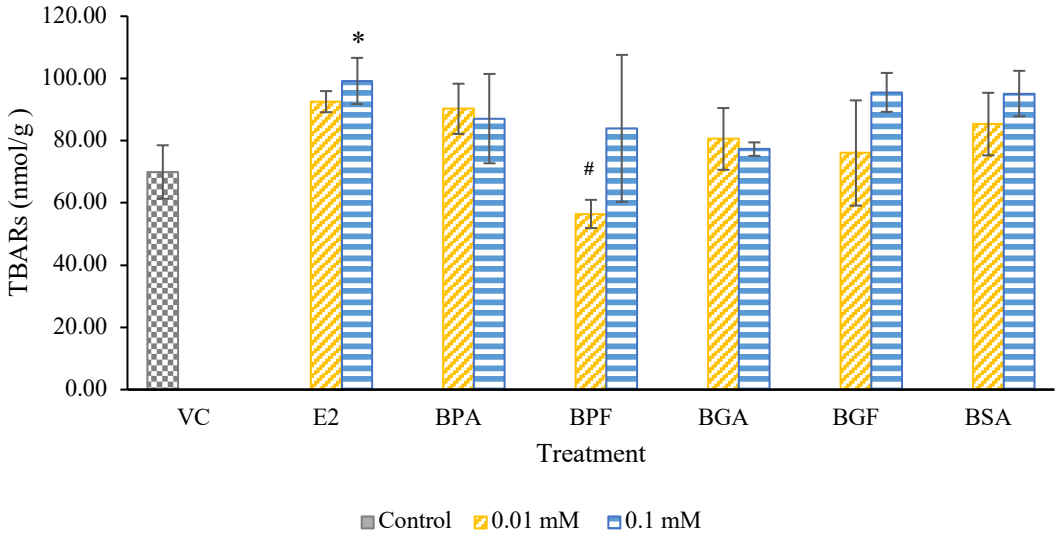

Figure A2: Impacts of E2, BPA, BPF, and three lignin-derivable monomers on TBARS values at two injection concentrations (0.01 and 0.1 mM).

Values are expressed as mean ± SD from two independent trials. * indicates a significant difference from VC ($p < 0.05$), and # indicates a significant difference from BPA at the same concentration ($p < 0.05$).