

Effects of Resveratrol Analogues on *Danio rerio* Mortality and Embryonic Development

Jesse Champer¹, Sarah Cushion¹, Sierra King¹,
Carlee Rushlow¹, Bertin Cenatus¹, and Lyndsay V. Rhodes^{1*}

Abstract - Phytoestrogens are commonly found in many plant-based food products, such as legumes and beans, and possess structures similar to estrogens that provides them with an affinity for estrogen receptors. As such, these compounds are able to alter the function of many estrogen-influenced systems including breast, bone, cardiovascular, and reproductive tissues. Studies on phytoestrogens have found both harmful and beneficial health impacts in human and animal models. Here, we focus on a class of phytoestrogens known as stilbenes, including resveratrol and pterostilbene, which have gained popularity as dietary supplements. The effects of stilbenes on embryonic development is an intriguing area of study due to their significant potential for disruption of estrogen signaling. The purpose of the present study was to expand the current scientific knowledge on how phytoestrogens, specifically stilbenes, affect the development and mortality of *Danio rerio* (zebrafish) embryos. The zebrafish was used as the animal model system for this research due to the wealth of existing data on developmental parameters, as well as the capacity of zebrafish for rapid and readily observable reproductive stages. Embryos were exposed to a series of stilbene compounds *in vivo* shortly after fertilization, and developmental endpoints and mortality were tracked throughout embryogenesis to functional maturity. Our data identified five stilbene compounds that display significant detrimental effects on embryonic growth and/or mortality rates, demonstrating the need for further studies on the safety of these compounds for human consumption at higher levels.

Introduction

Phytoestrogens are a diverse group of compounds found in many plant products commonly consumed by humans, such as legumes and beans (Desmawati and Sulastri 2019, Křížová et al. 2019, Murkies et al. 1998). The phytoestrogens share a common aromatic ring structure similar to estrogens and are able to directly bind and regulate estrogen receptors (ER), inducing both agonistic and antagonistic effects (Basu et al. 2018, Tham et al. 1998, Ye et al. 2019). Many of these compounds are able to freely traverse the cellular membrane due to their structure and relatively low molecular mass, allowing them to influence a variety of tissues throughout the body (Lasić et al. 2019, Liu et al. 2018, Ososki and Kennelly 2003). Another remarkable quality of phytoestrogens is their capacity to bind both ER-alpha and ER-beta estrogen receptor subtypes, resulting in a wide distribution of physiological effects throughout the body (Cederroth and Nef 2009, Lasić et al. 2019). However, most phytoestrogens have a greater affinity for ER (Hsieh et al. 2018). This is significant due to the differential distribution of ER throughout the body and differential gene regulation. As a result, phytoestrogens have the greatest impact where ER is most strongly expressed, including breast, cardiovascular, bone, and reproductive tissues (Hsieh et al. 2018, Patisaul and Jefferson 2010). The structural diversity of phytoestrogens also allows them to influence many estrogen-independent biological functions, such as protein synthesis, enzyme activity, and growth factor activity (Aldercreutz and Mazur 1997).

¹Department of Biological Sciences, Florida Gulf Coast University, 10501 FGCU Boulevard, South, Fort Myers, FL 33965. *Corresponding Author: Lyndsay Rhodes, lrhodes@fgcu.edu

Several studies have been conducted on the impact of phytoestrogens on animal and human health, and it has been found that these compounds can elicit both beneficial and harmful responses. Reported benefits include promotion of cardiovascular health, bone health, and management of menopausal symptoms, as well as anti-cancer effects (Desmawati and Sulastri 2019, Knight and Eden 1996). Although many potential benefits exist for the use of phytoestrogens, their capacity for endocrine disruption may also cause harmful effects when ingested at significant amounts. In many animal models, experiments have suggested that estrogen disruption during gestation leads to abnormal development of female reproductive structures, such as the ovaries, uterus, and mammary glands (Crain et al. 2008, Patisaul 2017). Likewise, evidence has also demonstrated negative impacts of estrogenic endocrine disruptors on male reproductive health, including diminished sperm quality and underdeveloped or undescended testes (Patisaul 2017, Santti et al. 1998).

The phytoestrogens of focus in this experiment are members of the biphenolic stilbene family. Many stilbenes are found in low doses in the average human diet (Ziauddeen et al. 2018). A major dietary contributor among natural stilbenes is the compound resveratrol, which has been identified in dozens of plant products including red grapes, blueberries, and many nuts (Ignat et al. 2011, Rauf et al. 2017). Several health advantages have linked to the dietary intake of resveratrol, such as beneficial antioxidant effects, cardiovascular protection, as well as anti-cancer and anti-aging properties (Fernandez-Mar et al. 2012, Rauf et al. 2017). Another notable stilbene is pterostilbene (Ptero), which is a resveratrol analog found in grapes, blueberries, and certain tree products (Cherniack 2012, McCormick and McFadden 2012). Though not as well studied, research on pterostilbene has indicated several potential health benefits, including antioxidant and anti-inflammatory properties, cardiovascular benefits, and anti-cancer effects on multiple cancerous tissue types (Estrela et al. 2013). Despite the various beneficial qualities mentioned, the affinity of stilbenes for estrogen receptors can also have a disruptive effect on normal endocrine function.

While dietary levels of phytoestrogens and stilbenes are relatively low individually, the popularity of natural supplements is significantly increasing in recent years (Pezzuto 2019). Due to being marketed as natural supplements, these products are not FDA regulated and dosages are not normalized. Most supplements available over the counter contain 95% or higher levels of pure stilbene, with recommended daily doses between 100mg and 500mg. The wide availability and absence of regulation of these supplements requires more empirical testing of the effects of these compounds on human health.

When approaching a laboratory design to test the effects of stilbenes on endocrine function and development, a suitable and experimentally advantageous organism must be used. Our study utilizes the *Danio rerio* (zebrafish) model of embryonic development (Howe et al. 2013a, b). The present experiment has been designed to characterize the impact of resveratrol analogues on various endpoints of embryonic growth *in vivo*, including mortality, malformations, and penetrance. The zebrafish embryos were exposed to a series of resveratrol analogues post-fertilization and observed for changes in mortality to demonstrate the impact of stilbenes on embryogenesis. Dose response studies were subsequently carried out for stilbenes demonstrating significant mortality effects to assess alterations of key developmental stages. These compounds were found to significantly alter mortality and maturity, including abnormalities in several characteristics of the embryos' external physiques, as well as delays in certain stages of development.

Material and Methods

Chemicals

Stilbene compounds (Table 1) were generously provided by Dr. Agnes Rimando at the US Department of Agriculture. All compounds were analyzed by proton (^1H) nuclear magnetic resonance (NMR) spectroscopy and gas chromatography–mass spectrometry (GCMS) to confirm identity and purity prior to biological analysis. *E/Z* isomers were determined by ^1H NMR, by observing $J = 16\text{--}16.5$ Hz (*E*) and $J = 12\text{--}12.5$ Hz (*Z*) for the vinyl protons, and isomers were confirmed pure ($> 99:1$ isomeric ratio) by gas chromatography with flame-ionization detection (GC-FID). ^1H NMR analysis was performed on a JEOL 400 MHz spectrometer in solutions of chloroform- d (CDCl_3). GC separations were performed on a Restek RTX-5MS column (30 m, 0.25 mm, 0.25 μm); 2 runs, 60 $^\circ\text{C}/\text{min}$, 2 mL/min, and 20 $^\circ\text{C}/\text{min}$, 3 mL/min. Compound weights were obtained on a Mettler Toledo MS105 microbalance (0.01 mg precision). All stilbene compounds were dissolved in dimethyl sulfoxide (DMSO) to stock solutions of 10mM and then further diluted to indicated concentrations. Final concentration of DMSO in embryo treatment conditions was 1 $\mu\text{L}/\text{mL}$. Stilbene compounds were stored at -20 $^\circ\text{C}$ and protected from light to avoid photodegradation.

Zebrafish husbandry

Wildtype zebrafish (mature adult) were obtained from Carolina Biological Supply and maintained in sex-specific 10-gallon tanks, containing no more than 12 fish per tank. The fish were sustained on a 12:12 light/dark cycle and fed a combination of brine shrimp, blood worms, and tetra flakes twice daily. All fish used in the experiment were cared for in accordance to the protocols approved by the Florida Gulf Coast University Institutional Animal Care and Use Committee (Protocol 1617–02).

Breeding

Zebrafish were placed in a small breeding tank at a ratio of 1 female to 1 male approximately 60 minutes after afternoon feeding. A 12:12 light/dark cycle was maintained overnight, and spawning was induced in the morning at the onset of light.

Embryo collection and treatment

After breeding, fish were returned to the sex-specific tanks. Embryos were collected with a strainer and washed thoroughly 3 times with embryo water (0.6 g aquarium salt per liter of deionized water). Embryos were transferred into a sterile 60 mm petri dish in embryo water

Table 1. Stilbene Compounds.

Stilbene Compound	Chemical Name
SB 1	(<i>E</i>)-1,3-dimethoxy-5-(4-nitrostyryl) benzene
SB 2	(<i>Z</i>)-1,3-dimethoxy-5-(4-nitrostyryl) benzene
SB 3	(<i>E</i>)-4-(3,5-dimethoxystyryl) aniline
SB 4	(<i>Z</i>)-4-(3,5-dimethoxystyryl) aniline
SB 5	(<i>E</i>)-methyl 4-(3,5-dimethoxystyryl) benzoate
SB 6	(<i>Z</i>)-methyl 4-(3,5-dimethoxystyryl) benzoate
SB 8	(<i>E</i>)-1,3-dimethoxy-5-(4-methoxystyryl) benzene
SB 9	(<i>Z</i>)-1,3-dimethoxy-5-(4-methoxystyryl) benzene
SB 15	(<i>E</i>)-4-(3,5-dimethoxystyryl) phenol (pterostilbene)
SB 16	(<i>E</i>)-5-(4-hydroxystyryl) benzene-1,3-diol (resveratrol)

(final concentration of 60ug aquarium salt/mL of water) and separated from non-viable embryos using a sterile transfer pipette. Viable embryos were distributed into 24-well plates, with 3–6 embryos in 1 mL of embryo water per well (final concentration of 60ug aquarium salt/mL of water). Final density of embryos per condition was determined by the number of total viable embryos available with each breeding. The number of embryos per condition was kept constant for all conditions within an independent trial. See Table 2 for number of embryos per trial. At 4–5 hours post-fertilization (hpf), embryos were treated with the stilbene compounds. Embryos were treated with 10 µM final concentration for initial screening experiments. Treatments for dose response assays ranged from 0.1 µM–5.0 µM final concentrations in embryo water. Water changes and re-treatment with stilbenes were conducted every 24 hours to maintain proper treatment concentration (avoiding effects of compound breakdown due to light and temperature) and to remove dead embryos and waste. Because the stilbenes are low molecular weight, removal of the embryo’s chorion was not necessary. Embryos were maintained at 28.6 °C for the duration of each experiment. See Figure 1 for illustration of treatment scheme.

Developmental endpoints and mortality

Zebrafish embryos were observed every 24 hours from initial stilbene exposure through 120 hpf for developmental endpoints and mortality. Developmental features that were evaluated include pericardial edema, yolk sac edema, and tail flexion, as well as fin fold defects and eye development. Endpoints were quantified in binary, in which abnormalities were scored as either absent or present. Percent penetrance was calculated via the number of abnormalities found for each developmental feature per total number of embryos. The number of dead embryos was recorded for each stilbene compound at all concentration levels and divided by the total number of embryos per condition to determine percent mortality. Mortality calculations were cumulative over the timepoints reported. Dead embryos were removed from treatment wells to minimize effects on remaining embryos.

Preservation of fry

After the 120 hpf time-points, living fry were euthanized following standard humane guidelines and stored in formalin at room temperature for future examination.

Statistical analysis

All experiments were conducted in a minimum of biological triplicates with internal duplicate wells (see Table 2 for N/trial and condition). Percent mortality or percent penetrance

Table 2. Number of embryos per condition per trial.

Trial	Number of embryos per well	Number of wells per treatment	Total embryos per trial
Screen T1	5	10	120
Screen T2	4	8	96
Screen T3	5	10	120
Total for screen			336
DR T1	3	2	150
DR T2	3	2	150
DR T3	4	2	200
Total for dose response			500

was calculated for each independent experiment. Values for replicate experiments were then averaged to determine overall change. Two-way ANOVA was run to determine significance with Dunnet post-hoc testing to determine significance between test conditions and control. All results presented are significant at 95% confidence or above.

Results

Select stilbenes induce mortality of zebrafish embryo in a dose dependent manner

Resveratrol, pterostilbene, and eight resveratrol analogues were screened to determine effects on zebrafish embryo in culture (Fig. 1). Embryos were treated with 10 μ M dose of

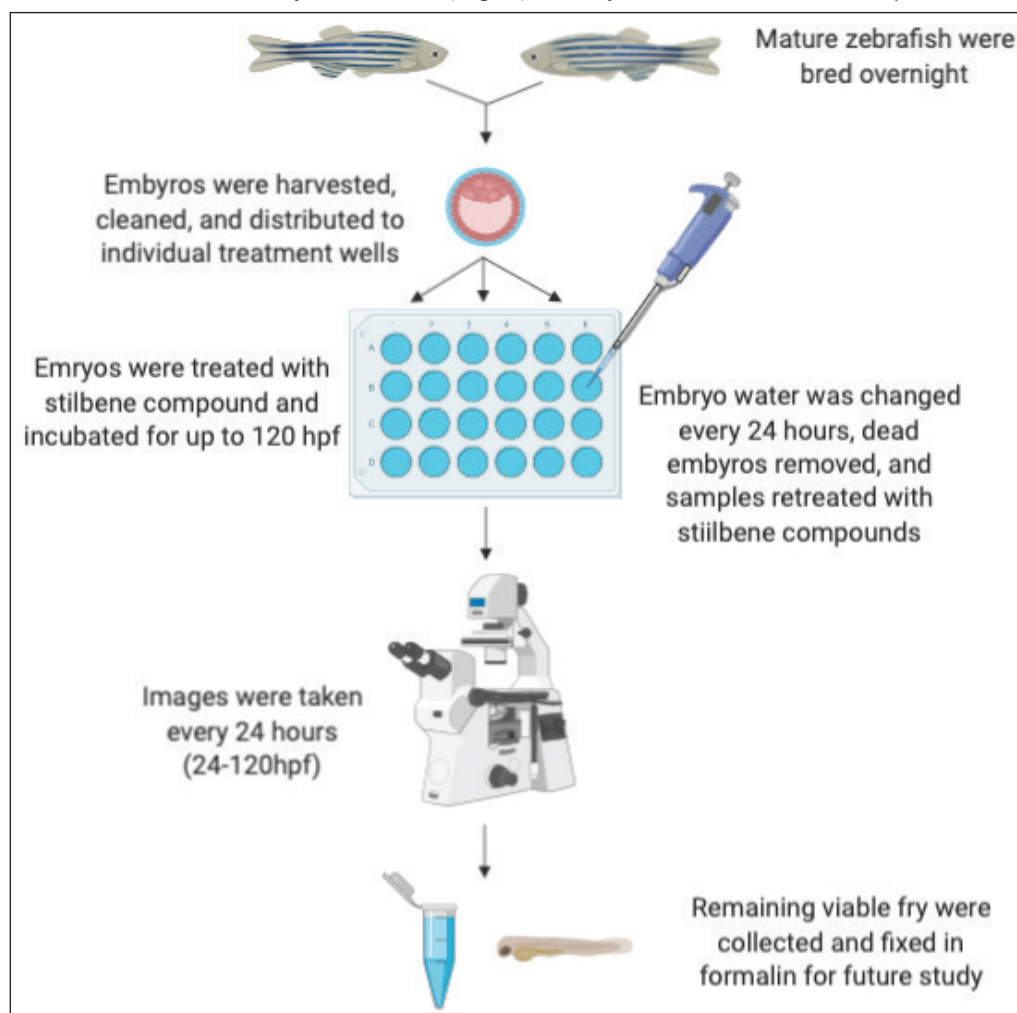


Figure 1. Experimental design illustration. This figure illustrates the overall experimental design and treatment scheme for reported experiments. Mature zebrafish were bred overnight in a separate breeding container maintaining a 12:12 light cycle. Within 1 hour of spawning, embryos were collected, washed, and non-viable embryo removed. Embryos were then distributed into 24 well plates in 1 mL of embryo water and treated with stilbenes at concentrations indicated for each experiment. Development and mortality were monitored every 24 hours. Embryo water was changed every 24 hours, deceased specimens removed, and embryos retreated with designated stilbenes to ensure stable conditions over the duration of the experiment. At the completion of the experiment, any remaining embryos were humanly euthanized and fixed in formalin for future study.

individual compounds within 4 hpf and mortality recorded every 24 hours. The average dose of resveratrol supplements ranges from 100mg to 500mg per day, corresponding to 1.43 to 8.33 mg/kg per day for the average adult human. The initial screening dose of 10 μ M equates to 2.28 mg/kg dosage of resveratrol in the zebrafish embryo and was chosen as a lower-to-mid dose starting point to test our compounds. As shown in figure 2A, stilbenes 3, 4, 6, 8, 9, and Ptero all induced death as early as 24 hpf above 50%. Stilbenes 4, 6, 8, and 9 demonstrated 100% mortality at 48 hpf, indicating a rapid effect on viability (Fig. 2A). Interestingly, the parent molecule resveratrol showed very little effect on embryo mortality, indistinguishable from control conditions, while pterostilbene showed moderate effects on mortality at the same dose (50–68%).

To determine differences in potency of these compounds, stilbenes found to be significantly active at 10 μ M (SB3, 4, 6, 8, 9, and Ptero) were selected for dose response experiments. Zebrafish embryos were harvested and treated within 4 hours of fertilization and exposed to individual stilbenes at concentrations of 0.1, 1, 2.5, and 5 μ M. Figure 2B demonstrates a clear dose-dependent effect of these active stilbenes with increasing doses (up to half the original dose) inducing mortality at all time points tested. Stilbene 9 showed high levels of mortality in embryos treated with even the lowest doses, indicating its enhanced potency. Pterostilbene, however, showed very little activity (less than 20% mortality) at these lower doses (Fig. 2B).

Stilbenes alter embryonic development early and persistently

Images were taken of developing zebrafish embryos at 24 hour intervals (24, 48, 72, 96, and 120 hpf) to determine if stilbenes effect not only mortality, but also normal development. Images were scored for altered eye development, fin fold development, heart or yolk sac edema, and tail flexion. General stage delay was also recorded. Figure 3A highlights these key deformities with representative images at the time points at which they were observed. One may note the range in appearance of each deformity and severity between each case.

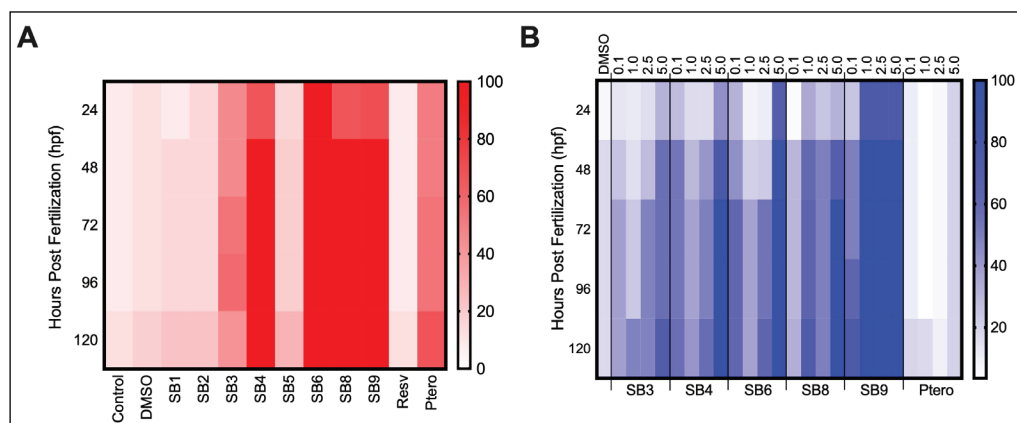


Figure 2. Stilbenes induce mortality in Zebrafish embryos in response to varying doses. (A) Zebrafish embryos (< 4 hpf) were exposed to the indicated stilbene at a concentration of 10 μ M for the duration of each experiment. Embryos were assessed every 24 hours to determine the percent mortality of each condition. White shading represents 0% mortality and increasing red saturation represents increasing percent mortality rates up to 100% for the darkest shade. (B) Zebrafish embryos were exposed to stilbene compounds in increasing doses. Each stilbene was administered at the following doses: 0.1 μ M, 1.0 μ M, 2.5 μ M, and 5.0 μ M. White shading represents 0% mortality and increasing blue saturation represents increasing percent mortality rates up to 100% for the darkest shade. All experiments were conducted with a minimum of three independent breeding experiments.

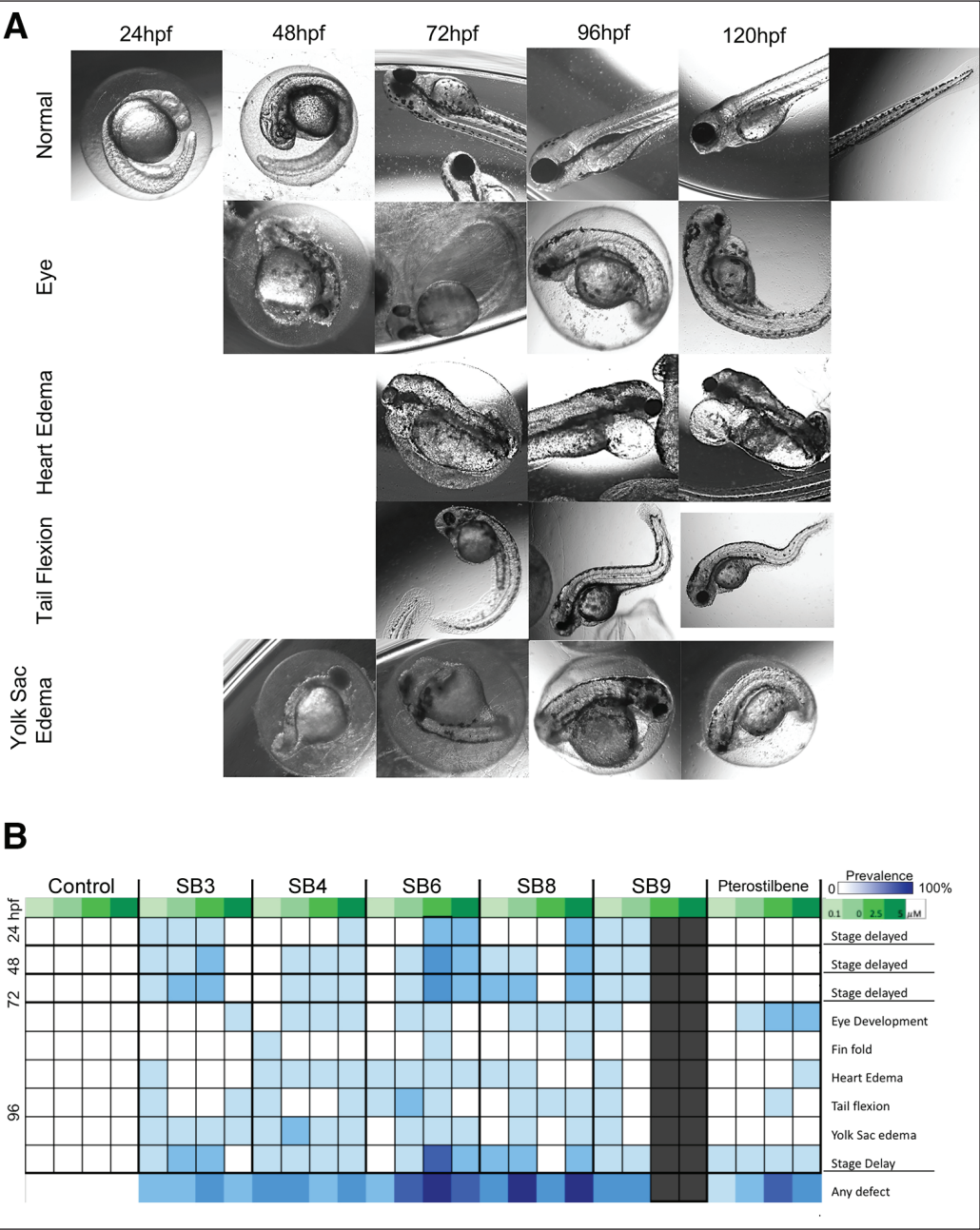


Figure 3. Stilbene-induced developmental abnormalities arise at early timepoints. (A) Representative images at 40X magnification for each time-point observed. Key abnormalities observed: eye development delay, heart edema, tail flexion, and yolk sac edema. Absence of image indicates this defect was not observed at that time point. (B) The varying shades of green boxes along the top of the grid indicate increased doses of stilbene from 0.1 μ M to 5.0 μ M. The blue boxes indicate penetrance of adverse effects per dose at each point in time. General stage delay was recorded between 24–72 hpf. In addition to stage delay, heart edema, yolk sac edema, tail flexion, eye development, and fin fold defects were recorded at 96 hpf. The proportion of embryos with deformities was recorded, with white indicating no deformities and darker shades of blue gradually increasing to 100% penetrance.

Thus, we scored our images based on the presence or absence of the deformity only, and not severity of deformity to decrease subjectivity.

Generally, we observed an increase in abnormal embryonic development in a dose- and time-dependent manner (Fig. 3B). This is particularly true for SB6, 8, and 9 with developmental abnormalities being more common at higher doses and later timepoints. While mortality was high for these compounds at the 10 μ M dose used in the screen, we did not observe many deformities at the highest doses, presumably due to the high toxicity of the compound at this dose (Fig. 2). In the dose-response experiments, complete mortality was not observed except for SB9, which demonstrated 100% mortality at 2.5 μ M and 5 μ M. To determine the overall effects of these active stilbenes on development, we totaled the presence of any deformity at any timepoint (Fig. 3B). Again, SB6 and SB8 demonstrated the highest abundance of abnormal developmental observations indicating higher potency of these compounds. However, SB9 is not to be discounted due to the high mortality rate compared to the other stilbenes tested (Fig. 2).

Select stilbenes elicit effects on embryonic development at low doses and early timepoints

The lowest observed adverse effect levels (LOAEL) for each stilbene at each time point was determined to define the potency of each compound on zebrafish embryonic development (Fig. 4). As expected, incidence of developmental abnormalities increases with time of exposure, with all stilbenes tested in the dose response showing significant levels of abnormalities at 72, 96, and 120 hpf, with the exception of pterostilbene. At the earlier timepoints (24–48hpf), the majority of stilbenes had a moderate to low LOAEL with only 2.5 to 5 μ M doses showing effects (Fig. 4). The only exception to this was again SB9 that showed significant effects on several developmental endpoints (yolk sac edema, stage delay) at the lowest dose tested (0.1 μ M). SB9 effects on development were persistent across all timepoints tested, with effects extending across all developmental endpoints observed (Figs. 3 and 4).

Discussion

Natural compounds are being explored more and more as mainstream medical remedies. These compounds are being developed by pharmaceutical companies and are also being marketed as dietary supplements with much more lenient regulation

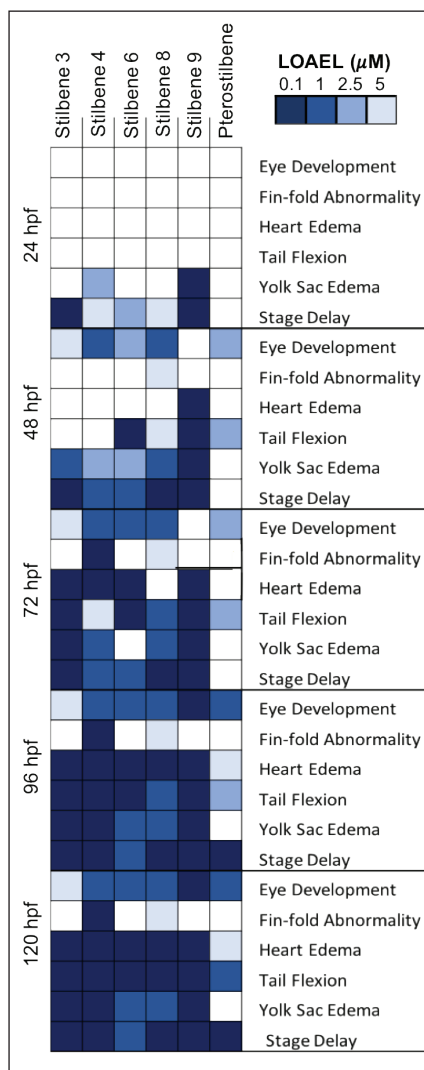


Figure 4. Select stilbenes induce developmental defects at low doses. The lowest observed adverse effect levels (LOAEL) were determined for each stilbene at 24, 48, 72, 96, and 120 hpf. Darker boxes indicate the presence of defects at lower doses, and lighter boxes indicate defects found at higher doses. All effects (shaded boxes) were found to be significant at $p < 0.05$.

than synthetic drugs. Therefore, these compounds are being used in unregulated amounts for long durations as well as being released into our environment through human excretion or manufacturing waste. Our study set out to determine the effects of a subset of resveratrol and its analogues on zebrafish embryonic development to shed light on the biological effects that may be yet unknown.

We observed stilbenes 3, 4, 6, 8, and 9 were the most lethal analogs of resveratrol screened at an initial dose of 10 μ M. The results of the subsequent dose response assays determined that SB9 caused 100% mortality at doses as low as 2.5 μ M at early timepoints, indicating the potency of this compound on zebrafish viability. The other stilbene compounds used in the dose response caused several deformities, specifically heart and yolk sac edema, at moderate concentrations and timepoints. The most substantial defects of the dose response were observed at 96 hpf and beyond, suggesting that longer exposure leads to increased severity of developmental abnormalities.

Resveratrol is being touted as a “miracle drug”, being studied for its effects on everything from metabolic disorders and obesity to cancer therapies. Research to develop more potent or specific analogues of resveratrol have led to the development of synthetic drugs, including those used in this study. And while these new analogues of resveratrol are showing potential as novel therapies in various systems (Mizuno et al. 2017, Pany et al. 2017, Paul et al. 2010) information on the safety of these compounds on normal processes is severely lacking. Additionally, the bioavailability of many natural compounds, including resveratrol, is very low, meaning that higher doses must be taken to elicit measurable effects (Lagoa et al. 2019). Since these are considered natural compounds, regulations on the proper dosing and frequency of use is not well regulated, and the safety of these long and sustained doses has not been adequately researched. The goal of the research presented here is to delineate between safe and potentially hazardous compounds to better inform therapeutic development, and caution administration of natural compounds to potentially susceptible patients, specifically pregnant women.

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