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Toxicological and behavioral responses as a tool to assess the effects of natural and synthetic dyes on zebrafish early life

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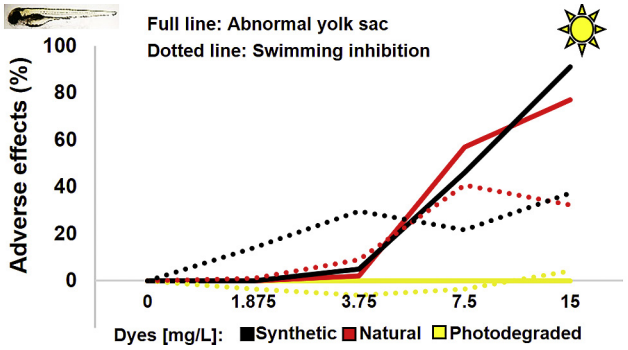
HIGHLIGHTS

- Natural and synthetic dyes induced malformation effects to zebrafish early life.
- Zebrafish larvae swam less when exposed to natural and synthetic dyes.
- Photodegraded natural dye did not induce any adverse effect in zebrafish early life.
- Behavioral assay has higher sensitivity towards malformations detection.
- A multiparameter approach promotes the assessment of ecological relevant effects.

GRAPHICAL ABSTRACT



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ABSTRACT

Organic dyes extracted from natural sources have been widely used to develop safety and eco-friendly dyes as an alternative to synthetic ones, since the latter are usually precursors of mutagenic compounds. Thereby, toxicity tests to non-target organisms are critical step to develop harmless dyes to environment and in this context, zebrafish early life stages are becoming an important alternative model. We aimed to assess the toxic effects of the synthetic dye Basic Red 51 (BR51, used in cosmetic industry), the natural dye erythrostominone (ERY, a potential commercial dye extracted from fungi) and its photodegradation product (DERY), using zebrafish early life assays. Developmental malformations on embryos and behavioral impairment on larvae were explored. Our results showed that embryos exposed to BR51 and ERY exhibited a large yolk sac (LOEC = 7.5 mg L^{-1}), possibly due to a deformity or delayed resorption. ERY also induced pericardial and yolk sac edemas at high concentrations (LOEC = 15 and 30 mg L^{-1}, respectively). Moreover, larvae swan less distance and time when exposed to ERY (LOEC = 7.5 mg L^{-1}) and BR51 (LOEC = 1.875 mg L^{-1}). The lowest larvae locomotion have been associated with impairment of the yolk sac, important tissue of the energy source. Interestingly, DERY did not affect neither development nor behavior of zebrafish, showing that ERY photodegradation is sufficient to prevent its toxic effects. In conclusion, both natural and synthetic dyes impaired development and

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behavior of zebrafish early life, therefore, a simple treatment of the natural dye can prevent the aquatic life impact.

1. Introduction

Synthetic dyes have been widely used by cosmetic, food, pharmaceutical and textile industries due to their low cost, ease of obtainment and stability (Jacobson and Wasileski, 1994). Among these, azo dyes represent the largest and most versatile group, but after cleavage of its azo bond, some of them can generate aromatic amines that are potentially mutagenic or carcinogenic (Reid et al., 1984; Chequer et al., 2001; Leme et al., 2015). Despite the fact that studies have shown the mutagenic potential of the azo dye Basic Red 51 (BR51) (Zanoni et al., 2014), its high production by cosmetic industries still ranges from 0.1 to 0.5 t per year (IARC, 2010). Taking into account several studies, the European Commission has reduced the number of approved dyes for human consumption purposes (SCCNFP, 2001; EC Directive 2002/61/EC; EC 2015/1190/EC) leading to a worldwide interest in the development of natural dyes that could be safe to humans and to the environment (Velmurugan et al., 2010; Caro et al., 2012; Lopes et al., 2013; Punrattanasin et al., 2013).

Natural dyes extracted from microorganisms are the most interesting group due to the possibility of their scale-up by bioreactor processes, allowing a fast and cheap production that requires a small area (Mapari et al., 2009; Dufossé et al., 2014). Some colorants extracted from fungi, such as *Monascus* pigments, Arpink Red™, riboflavin, lycopene and β-carotene, are already marketed (Dufossé et al., 2014), and some studies have highlighted the successful use of fungal pigments on leather dyeing (Velmurugan et al., 2010). Moreover, several natural dyes, such as flavonoids, betalaines, tetrapyrroles, and tetraterpenes (Shahid et al., 2013; Dufossé et al., 2014), present beneficial additional biological activities, such as anti-inflammatory, anti-viral, anti-fungal, antimicrobial and anti-cancer properties, that might be important regarding human health status (Kittakoop et al., 1999; Xiao and Zhong, 2007; Anantharaman et al., 2014; Silva et al., 2014).

Among groups of natural dyes, naphthoquinones are an alternative source of red dyes that can be extracted from fungi. Erythrostominone (ERY) is one example of naphthoquinone dye that has been already isolated from the *Gnomonia erythrostoma* (Cross et al., 1970, 1972) and *Cordyceps unilateralis* (Kittakoop et al., 1999; Unagul et al., 2005; Prathumpai et al., 2007) fungi. For the first time, ERY was isolated from an endophytic fungus found in the Brazilian red mangrove (*Rhizofora mangle*). Previous studies have shown that ERY could be considered non-genotoxic and non-cytotoxic when applied topically on human dermal equivalents (Abe et al., 2015). Moreover, ERY showed antimalarial activity (IC50 10.1 μg mL^{-1}) and induction of cytotoxic effects on tumor cells lines (Kittakoop et al., 1999). Despite previous studies showing its non-cytotoxicity to human cells, ERY has a high bioactivity potential and can be harmful to non-target organisms if it reaches water resources through industrial and domestic effluents. Textile industries, for example, can discharge over 15% of their dyes into effluents (Nojavan et al., 2013). Thus, those wastewaters are subject of Directive Regulations (EC 2000/532/EC; EC Directive 2006/12/EC; EC 2014/955/EU) becoming a critical step on dye manufacturing to develop harmless dyes to the environment.

Within this context, the identification of safe dyes to the environment is relevant to prevent harmful effects to non-target organisms exposed to them. Zebrafish (*Danio rerio*) has become a major model in toxicology with well-characterized early development and transparent embryos allowing detailed observation of organogenesis, besides being an inexpensive model with easy maintenance (Braunbeck et al., 2005; Lammer et al., 2009; Knobel et al., 2012; Scholz, 2013). Fish embryos toxicity (FET) assay was proposed by Nagel (2002) in order to assess morphological changes as an alternative to fish acute tests. Recently, behavioral approaches have been suggested as a reliable tool to investigate zebrafish locomotion that is considered an early-warning biomarker of adverse effects, with increasing sensitivity and predictability when compared to traditional toxicity tests (Macphail et al., 2009; Padilla et al., 2011).

Our current research study aimed to investigate the toxic effects of BR51, ERY and also its photodegraded product (DERY) to zebrafish early life stages using the following parameters: survival, development and behavior. For that, FET was performed using zebrafish embryos, in order to investigate effects of BR51, ERY and DERY on their survival and malformations during development, such as edema, yolk sac deformities and retarded growth. Then, behavior was determined by locomotor activity, measuring the distance and time spent swimming, as well as velocity of zebrafish larvae exposed to dyes.

2. Material and methods

2.1. Instruments

HPLC-DAD-MS analysis were obtained on an Acquity™ UPLC system equipped with a quaternary pump and an automatic injector coupled to PDA UPLC AcquityTM and Xevo TQ-S mass spectrometer with Z-spray orthogonal ionization source ESI (electrospray) (Waters®) using Ascentis® Express C18 column (5.0 cm × 2.1 mm, 2.7 μm) (Supleco). The MS parameters were as follows: 3.2 kV capillary voltage, 30 V cone voltage, 350 °C desolvation temperature, 600 L h^{-1} gas flow and 5.0 Bar nebulizer gas. HRMS were obtained by direct infusion using a syringe pump operated with 300 μL h^{-1} flow rate, coupled with a mass spectrometer micrOTOF Q-II (Bruker®), using the follow parameters: 3.5 kV capillary voltage, 500 V end plate offset, 4 L min^{-1} dry gas and 180 °C dry temperature. NMR spectra were obtained at 500 MHz for ^{1}H and 125 MHz for ^{13}C on Bruker® AVANCE DRX500 using methanol-d_4 (CD_3OD) as a solvent.

2.2. Production of erythrostominone

Endophytic fungus LC01-A strain (unknown species in identification phase) isolated from *R. mangle* was kindly provided from Coleção de Microrganismos de Importância Agrícola e Ambiental (CCMA, EMBRAPA, Brazil). The fungus LC01-A was cultivated in 500 mL Erlenmeyer flasks containing 150 mL PD (potato-dextrose) medium at 30 °C under shaking conditions (200 rpm) for 15 d. After this period, the crude extracts were obtained by simple liquid culture filtration followed by liquid-liquid extraction with ethyl-acetate (1:1, v/v). Solvent was