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Assessment of Developmental Toxicity of Indigo (*Indigofera tinctoria* L.) - An Industrially Important Dye in Zebrafish (*Danio rerio*)

Sasi Bhushan and Siva Ramamoorthy*

School of Bio Sciences and Technology, Vellore Institute of Technology, Vellore - 632014, Tamil Naud, India
*Corresponding Author: siva.ramamoorthy@gmail.com

Abstract

Chemically synthesized dyes have been used, mainly in textile industries for dyeing textile fabrics. Even though natural dyes have had a long history of being used to dye textiles, their importance and use have gradually reduced due to superior color fastness and their synthetic counterparts' cost-effectiveness. However, it can cause harm to the environment (water) and its associated life (mainly aquatic) if not disposed of properly. Certain synthetic azo dyes, owing to their detrimental effects on the environment, have since been banned. Indigo, obtained primarily from *Indigofera tinctoria* L. is one of the first dyes to be used by mankind and is still in use. Synthetic Indigo is predominantly used and preferred over natural Indigo because synthetic Indigo gives a better-finished look, and natural Indigo yield is too low to meet the denim industry's demands. In this study, natural Indigo extracted from the leaves of *I. tinctoria* L., and synthetic Indigo were characterized using FTIR and UV spectroscopic techniques. The develop-mental toxicity of the dye was studied on Zebrafish (*Danio rerio*) embryos. Lethal concentrations, abnormalities caused by the dye and morphological changes of the embryos have been studied. Toxicity levels were evaluated by calculating the LD_{50} values on the embryonic stages at 24h, 48h, and 72h. Morphological changes were noted using microscopic observations. Sublethal effects (spinal curvature, loss of pigmentation of the embryos) were observed in higher concentrations of the synthetic dye as well as the natural dye, but in lower concentrations, there was normal development of the Zebrafish embryos. In light of the study, there is a need for proper disposal of the by-products (during the production process) and the dye itself.

Keywords: Denim, Developmental toxicity, Indigo dye, Spectroscopy

Introduction

Dyeing is a primitive art that majorly involves the use of extracts from fruits, berries (1). Indigo has a long history of being used as a textile dye (2). Natural Indigo, extracted primarily from *Indigofera tinctoria* L., (Fabaceae) grown in India, was the most traded commodity in the old World (3). Textile fabrics dyed with natural Indigo held a special place in ancient civilizations (4). The blue colour imparted by Indigo on fabrics was far greater than any other dyestuff, and the dye was only imported from India (5). Indigo imparts colour only to the fabric's surface, not to the inner layers, and the dye has moderate to fast high lightfastness. Indigo has been one of the most important dyes in the dyeing world since antiquity (3). Indigo has sustained its importance because of its use to dye denim jeans. The unique ability of Indigo to achieve wash-down effects after repeated washing while retaining the freshness of the colour makes the dye important in the modern contemporary World (6).

The extraction of natural Indigo is laborious, time-consuming, and expensive. Natural Indigo production could not meet up

with the textile industry's requirements, mainly for the manufacture and dyeing of denim jeans. Natural Indigo was completely replaced by synthetic Indigo, owing to the demand and popularity of denim jeans during the Industrial Revolution. During the chemical synthesis of dyes, Indigo included by-products from the process and the dye is generally discharged into water-bodies such as lakes and rivers (7). These pollutants get mixed with the water, affecting the environment and the aquatic life associated with such water-bodies. Some of the textile dye effluents are carcinogenic and forms recalcitrant compounds in the aquatic environment (8).

Toxicological studies of extracts of pigment-yielding plants have been reported in earlier animal models. For instance, dibutyl phthalate from *Rubia cordifolia* fruits (9). However, there has been little to no studies on the developmental toxicity effects of Indigo, particularly in larval-embryos of Zebrafish (*Danio rerio*). Indigo has been observed to cause skin irritation after repeat and long exposure (10). Zebrafish is a small freshwater fish and a model organism for toxicological, developmental and genetic studies (11). The embryos of zebrafish are transparent and the developmental stages can be clearly observed under magnification under a light microscope. Zebrafish is an ideal model organism because it is easy to maintain and breed. Toxicological studies of drugs and other compounds are particularly easy because it can be directly added to the water. Zebrafish is mainly preferred because of its rapid development and large sample size obtained by breeding (12).

In this study, the extraction of Indigo from the leaves of *I. tinctoria* L. has been optimized and the dye characterized using UV-Visible spectroscopy and FTIR spectroscopy. The toxicity effects, using zebrafish as a model, of naturally extracted Indigo has been compared with that of synthetic Indigo.

Materials and Methods

Extraction of indigo dye:

Leaves of *Indigofera tinctoria* L. were collected, dried, and cut into small pieces and fermented in water overnight. The pH of the fermented liquor was adjusted to 9.0 and 11.0 using 1N NaOH. The liquor was then air oxidised for 15 min and boiled for 10 min to remove impurities. Indigo powder was obtained after centrifugation at 5000 RPM for 10 min and subsequent drying in a hot air oven at 50˚C (13, 14). Synthetic (Standard) Indigo was purchased from Sigma-Aldrich.

Characterization of Indigo Dye:

Indigo was characterized using UV-Visible spectroscopy and FTIR spectroscopy. 1 mg Indigo powder extracted from *I. tinctoria* L. was dissolved in 1 ml of 0.1% DMSO and used for UV-Visible spectroscopic analysis. The UV-Visible spectrum was recorded in the range of 200 – 800 nm (14) using a Cary 3500 UV-Vis Spectrophotometer (Agilent, Santa Clara). 5 mg of Indigo powder was directly taken for FTIR analysis with Cary 630 FTIR spectrometer (Agilent, Santa Clara). FTIR spectroscopy of Indigo was measured using Attenuated Total Reflectance (ATR) method. Both the UV and FTIR measurements were taken in the Central Instrumentation Facility, Vellore Institute of Technology.

Fish husbandry and embryo collection:

Wild-type Zebrafish (*Danio rerio*) were obtained and bred in the laboratory. The fishes were kept for spawning early in the morning and the embryos were collected 3 hours later. Viable embryos were selected and washed completely with E3 media (5.0 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) [15]. Healthy embryos, 6 hours post-fertilization (hpf) were selected with observations under a light microscope.

Dye exposure of zebrafish embryos:

Healthy fertilized embryos at 6hpf were transferred to a 24-well culture plate. 12 embryos per well were taken and with different concentrations of the Indigo dye solutions (Natural Indigo, extracted from *I. tinctoria* L. and synthetic Indigo, purchased from Sigma-Aldrich dissolved in 0.1% DMSO) in triplicates (0.5 µM, 2.5 µM, 5 µM, 12.5 µM, 25 µM and 50 µM). 0.1% DMSO was used as control. The experiment was carried out for 72 h. At 24 h intervals (24 h, 48 h and 72 h), the viability of the embryos was checked and counted to calculate

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the viability rate. The embryos' morphological characteristics were examined under a light microscope at 4 times magnification at the specified time intervals and photographed.

Results and Discussion

Extraction of indigo dye

The yield of Indigo dye was found to be better when the pH of the liquor was adjusted to 11.0 (Table 1). The yield of Indigo, when the pH of the fermented liquor was adjusted to pH 9.0 was around 1 g per 10 g of leaves (dry weight), whereas there was a 60% increase in the yield of Indigo when the pH of the fermented liquor was 11.0, yielding approximately 1.7 g of Indigo per 10 g of leaves (dry weight). *I. tinctoria* L. contains the Indigo precursor, indican. Indican is converted to indoxyl through fermentation by the action of an enzyme, β-glucosidase present in the leaves of the Indigo dye-yielding plant [6]. Two molecules of indoxyl combine to form Indigo by oxidation (Figure 1).

Table 1: Effect of pH on the yield of Indigo extracted from the leaves of *I. tinctoria* L.

pH of the fermented liquor	Indigo yield* (g)
9.0	1.06 ± 0.15
11.0	1.71 ± 0.08

*yield given per 10 g of leaves dry weight

Characterization of indigo dye by uv-visible spectroscopy and ftir spectroscopy

The Indigo dye extracted from the leaves of *I. tinctoria* L. was characterized by UV-Visible and FTIR spectroscopy. The UV-Visible absorption spectra were obtained using a Cary 3500 UV-Vis spectrophoto-meter. Measurement of the absorption spectra was done in the range of 200 – 800 nm. The maximum absorption peak of the extracted, natural Indigo was at a wavelength of 550 – 700 nm [16] in the visible range (Figure 2).

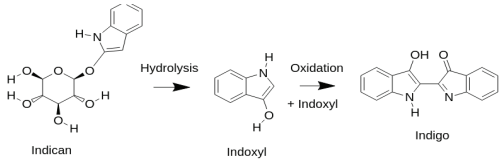

Figure 1: Extraction of Indigo from the leaves of *I. tinctoria* L. by fermentation (hydrolysis) followed by oxidation by air

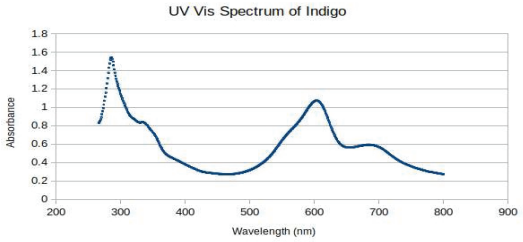

Figure 2: The UV-Visible spectra of Indigo with maximum absorption spectra at 550-700 nm.

The FTIR spectra (Figure 3) confirmed the presence of primary N-H and secondary amines at 3100-3500 cm^{-1}, aromatic C=C at 1600 cm^{-1}, aromatic C-N at 1000-1350 cm^{-1}, and aromatic C-H at 690-900 cm^{-1}.

Toxic effects of indigo on zebrafish embryos

Natural Indigo, extracted from the leaves of *I. tinctoria* L., and synthetic Indigo, purchased from Sigma-Aldrich were used in this study to assess their toxicological effects on Zebrafish embryos. Indigo carmine, a disulfonate derivative of the Indigo dye also has major industrial applications [17], however, it has shown toxicological effects on mice models [18]. But there have been no studies on the effects of Indigo on mice or zebrafish models. Synthetic Indigo has shown lethal effects on the developing embryos of zebrafish at a concentration of 50 µM and sublethal effects at other concentrations (0.5 µM, 2.5 µM, 5 µM, 12.5 µM, 25 µM). Sublethal effects were also observed in the developing embryos in natural Indigo, but there were no lethal effects observed. Control embryos grown in 0.1% DMSO showed no abnormalities or any toxico-logical effects (Figure 4).

The experiment which was done for 72 h, thrice the viability of the embryos was checked at 24 h, 48 h and 72 h intervals. The developing embryos were taken and observed under a microscope to check for any abnormalities or proper growth at every interval. If present, the dead embryos were separated from the live and healthy ones as the dead embryos tend to

secrete bodily fluids that could hinder the growth of the normal embryos. Zebrafish embryos were found to be dead by heart explosion (HE) at 72 h in the samples with synthetic Indigo (Figure 5). Abnormalities such as spinal curvature (SC), enlarged pericardial edema (PE) were observed in embryos in the presence of synthetic Indigo as well as natural Indigo (Figure 6). However, the extent of the damage was greater in the synthetic Indigo samples when compared to that of the natural Indigo samples.

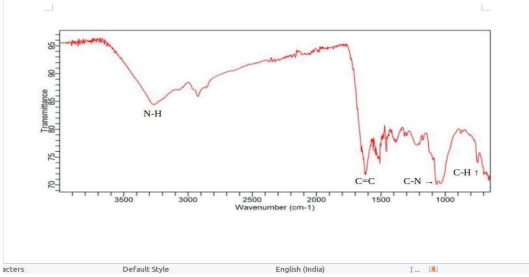

Figure 3: The FTIR spectra shows characteristic peaks of Indigo, 3100-3500 cm^{-1} as primary N-H and secondary amine, 1600 cm^{-1} as aromatic C=C, 1000-1350 cm^{-1} as aromatic C-N, 690-900 cm^{-1} as aromatic C-H

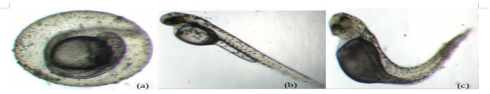

Figure 4: Control zebrafish embryos at (a) 24 hpf, (b) 48 hpf, and (c) 72 hpf

Figure 5: Zebrafish embryos grown with natural Indigo (50 µM) are normal at (a) 24 hpf and (b) 48 hpf, but shows abnormality (SC- Spinal Curvature) at (c) 72 hpf

Figure 6: Zebrafish embryos grown with synthetic Indigo (50 µM) have normal growth until (a) 24 hpf but begin to show abnormalities (SC- Spinal Curvature and enlarged PE Pericardial Edema) at (b) 48 hpf leading up to death (by HE- Heart Explosion) at (c) 72 hpf

Synthetic Indigo dye needs to be treated before being properly discharged. In addition to the dye, the waste products from the chemical process also need to be treated before released as wastewaters. Natural dye did not have drastic lethal effects when compared with its synthetic counterpart but did have some sublethal effects on the developing embryos. This study clearly shows the toxic effects of Indigo on developing embryos of zebrafish. When the synthetic Indigo dye, produced by chemical means, is dumped into water-bodies containing aquatic life forms (zebrafish included). The by-products and other pollutants can cause toxic effects and lead to disturbances in the environment. Certain chemical azo dyes have been known to cause harmful effects to humans and to the environment when dumped into water-bodies, and have since been banned (2, 19).

Conventional wastewater treatment techniques are quite ineffective in treating dyes and its associated by-products because of the chemical stability of those pollutants (7, 20). When mixed in the water, the dyes and the pollutants are taken up by the aquatic life forms, which ultimately leads to various abnormalities. In addition to the harmful effects, the dye also changes colour of the water, which leads to unpleasant perception. Techniques like nanofiltration, reverse osmosis, electrodialysis (Physical methods), coagulation, flocculation (Chemical methods) and bacterial degradation, decolourization by microbial cultures, etc., bioremediation can be employed to treat the dyestuff and its associated effluents.

Natural Indigo, which has been in use for centuries for dyeing textile fabrics, can since be used to dye textile fabrics instead of its synthetic counterpart. Although the extraction and application of natural Indigo require labour and is expensive when compared to synthetic Indigo. Natural Indigo is still being extracted and employed on a small scale in India, Central America, and Africa. The revival of natural Indigo with various biotechnological techniques like molecular cloning and plant tissue culture will indirectly help the environment.

Conclusion

Natural Indigo has been extracted from the leaves of *I. tinctoria* L. and the pH of the fermented liquor plays a role in the oxidation process and in the stability and the yield of the Indigo dye. The yield of Indigo was at least 60% greater when the pH of the fermented liquor was 11.0. The extracted dye has been characterized by UV-Visible spectroscopy and FTIR spectroscopy. The maximum absorption spectra obtained at 550-700 nm from UV-Visible spectroscopy and the peaks obtained at 3100-3500 cm^{-1}, 1600 cm^{-1}, 1000-1350 cm^{-1}, and 690-900 cm^{-1} from FTIR spectroscopy confirm the presence of Indigo in the extracted sample. Naturally extracted Indigo and synthetic Indigo showed toxicological effects in zebrafish models. Synthetic Indigo showed lethal effects and sublethal effects like spinal curvature and enlarged pericardial edema in the embryos. However, natural Indigo showed only sublethal effects in the embryos but did not cause any lethal effects. Synthetic dyes along with their associated pollutants, are generally discharged into rivers and lakes without any proper treatment which ultimately leads to toxicological effects in the life forms present in the water-bodies. Proper dye treatment techniques are to be used before being discharged into the environment. Current techniques like decolourization, bioremediation can be used for the treatment of synthetic dyes. Natural dyes can also be used instead of synthetic dyes. Natural dyes do not cause harm to the environment and living things to the extent of synthetic dyes.

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