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Original Research Article

Synthesis of a Novel Chemical J5 brown Dye for Staining Specific Bone Histological Sections in Green Swordtail Fish (*Xiphophorus hellerii*)

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ABSTRACT

The creation of aromatic pigments in 1859 revolutionized the dye industry, with azo dyes emerging as the most significant class due to their versatility, cost-effectiveness, and widespread applications in textiles, food coloring, and medical fields. The present study aimed to synthesize and evaluate J5 brown, a novel azo dye, for staining specific bone histological sections in green swordtail fish (*Xiphophorus hellerii*). The dye was synthesized using the diazotization and coupling reaction. Nuclear magnetic resonance (NMR) spectrometry, infrared spectrometry, and mass spectrometry (MS) were used to confirm the structure of the dye. Histological sections of the fish samples were prepared, and the dye was evaluated for its efficacy in staining fish bone tissues. The synthesized J5 brown resulted in a brown powder with a yield of 87% and a melting point of 162–164°C. When the dye's absorption properties were evaluated over a range of pH values (2–12), the results revealed maximum absorption at 430 nm between pH 2 and 8 and at 350 nm between pH 9 and 12. Solvent polarity also influenced the dye's absorption, with notable redshifts observed in polar solvents and water. J5 brown dye effectively stained fish bone tissues, outperforming Alizarin Red in highlighting bone structures with approximately 80% accuracy compared to traditional bone stains. The present study's findings revealed that the novel dye offers a cost-effective, stable alternative for bone tissue staining, with potential applications in both clinical and research settings. Further studies will explore its suitability for staining other tissues and its toxicity profile.

Keywords: Azo dye, Staining, Histological sections, *Xiphophorus hellerii*, J5 brown.

Introduction

William Henry Birkin created the first aromatic pigments in 1859 while attempting to make a drug for malaria and noticed that these pigments turned coloured. This marked the beginning of the scientific revolution in the dye industry.[1] Organic substances with the azo group (-N = N-) are known as azo dyes.[2] The azo dyes in their composition also contain groups called auxochromes, such as -NH2, -OH, -COOH, -NO2, and -NH2, which increase the appearance of pigment colour.[3] The diazonium formation method and the coupling reaction are suitable in the preparation of the azo dye, Jamudiazene Brown, because of their recurrent credibility in previous studies and the simplicity and low risk of these methods.[4] The most significant class of textile pigments are azo compounds, which are also highly sought after because of their low cost and ease of application.[5] The most significant class of industrial pigments, azo pigments are used extensively in the food colouring, industrial fiber, and wool textile dyeing industries.[6] Approximately 65% of the commercial pigment market is composed of azo pigments.[7] These organic dyes play a crucial role in distinguishing animal tissues and have diverse applications in medical and clinical settings.[8] The techniques used to prepare dyes vary depending on the specific objectives and the laboratory's requirements.

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These dyes can be analyzed both analytically and visually, with slight modifications to standard procedures.[9] Dye binding to bone tissue occurs through an adsorption process influenced by the chemical composition of the dye, particularly the presence of heteroatoms like nitrogen and oxygen, which possess lone pairs of electrons. These atoms, along with the aromatic rings in the dye, facilitate its attachment to the bone tissue. The adsorption process can be explained by two mechanisms. Physical adsorption involves the action of Van der Waals forces between the histological structure of the bone and the adsorbed ions, atoms, or dye molecules. In contrast, chemical adsorption entails synergistic interactions, which may be covalent, ionic, or a combination, occurring between the bone tissue and the ions, atoms, or molecules of the dye.[10] To enable the dye to bind to bone, the lone pairs of electrons on the heteroatoms in the dye form a synergistic bond with the mineral atoms, particularly calcium, which is a key component of bone tissue.[11] The chemical and physical characteristics of J5 brown dye, as discussed in this study, have prompted its evaluation as a novel chemical dye for staining animal tissue, offering a potential alternative to traditional pigments used for this purpose. The prepared pigment is both innovative and distinctive, as demonstrated by the results of the present study. Its applicability to various tissue types in the body highlights its potential as a valuable tool in clinical laboratories. Specifically, it can be used to identify calcium deposition sites in bone and may serve as a reliable pigment for detecting certain bone diseases or assessing stages of bone development. The present study was conducted to synthesize and evaluate a novel chemical dye, J5 brown, for effectively staining specific bone histological sections in fish to enhance visualization and microscopic analysis.

Materials and Methods

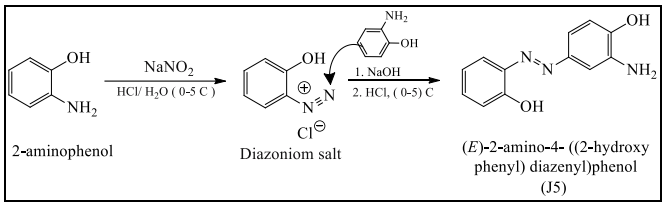
Sources of chemical reagents used and instrumentation

Solvents and reagents were purchased from reliable businesses like the German company, Merck, and the American company, Aldrich. A SHIMADZU FT-IR 8400S device from China was used to record

infrared spectroscopy results with 1% KBr disks. The melting points of the prepared vehicles were determined using a Chinese-made Thermo Scientific 9100 device. A GENWAY-6305 spectrophotometer, also Chinese-made, was employed to analyze the visible spectra. The mass spectra of the prepared compound were measured with a German-made spectrometer (Agilent Technology HP) utilizing electron impact (EI) technology at 70 eV. Proton nuclear magnetic resonance (^{1}H NMR) was conducted using a Bruker AVANCE-DRX spectrometer (German-made) operating at 400 MHz, with Tetramethylsilane (TMS) as a reference. Measurements of pH were performed using a Bremen pH scale device (Model L Puls Munchen) from China. Histological sections were sliced using a German-made rotary microtome (Leica Jung RM2035) and analyzed with a Leica DM500 light microscope, also German-made.

Synthesis of J5 Brown dye

Diazation and coupling, the approved methods for making azo dye, were used to prepare the dye.[12] Ortho-aminophenol 0.006 mole, 0.456 g of $NaNO_2$, and a 1.8% w/v solution of NaOH and 2.1 ml of HCl were required. The procedure for the synthesis of the azo compound, J5 brown, is outlined in Scheme 1.



Scheme 1: Preparation of the azo J5 brown dye ((E)-2-amino-4-((2-hydroxyphenyl) diazenyl) phenol)

Preparation of histological sections

Histological sections of the fish samples were prepared according to the procedures described by Russell *et al*. (2022).[9] The tissue samples were prepared by initially soaking them in a 10% formalin solution for 24 hours, followed by rinsing with ion-free water to remove the fixative. The samples were then stored in 70% ethanol. To prepare them for paraffin embedding, the samples were dehydrated through a graded ethanol series (80% and 90% for one hour each, then 100% for three hours). Clearing was performed using xylene for 2–5 minutes, after which the samples were placed in glass containers with paraffin wax and heated in an oven at 58°C. The samples were soaked in paraffin wax for approximately four hours before being embedded in special molds. After the paraffin blocks were trimmed, the samples were sectioned at a thickness of 7 micrometers using a rotary microtome and transferred to a water bath at 40–45°C. This process allowed the sections to expand and flatten, after which they were adhered to clean glass slides.

Preparation of solutions

A 0.001 M solution of J5 brown was prepared, followed by the preparation of solutions with pH values ranging from 2 to 12. Standard solutions of J5 brown at a concentration of 0.00008 M were then prepared and tested at various pH levels between 2 and 12. The absorption of these solutions was measured over a wavelength range of 320 to 470 nm. To assess the effect of different polar solvents, a standard solution of J5 brown (0.00008 M) was prepared using various solvents (n-hexane, 1.4-dioxane, chloroform, acetone, ethanol, methanol, dimethyl sulfoxide (DMSO), and ion-free water), and absorption was measured over a wavelength range of 330 to 460 nm.[13]

Staining of histological sections

The histological sections were stained following the procedure described by Burstone (1959).[14] To prepare the samples, the dye was dissolved in 99.9% high-purity ethanol. The glass slides with tissue sections were then soaked in xylene for 15 minutes (twice). Next, the sections were immersed in 100% ethyl alcohol for 10 minutes (twice), followed by a 5-minute soak in 90% ethyl alcohol and a 3-minute soak in 70% ethyl alcohol. The sections were quickly dipped in distilled water for 2 minutes. The slides with tissue sections were then placed in J5 brown dye for approximately 30 minutes. After staining, the slides were briefly dipped in hexane for 2 seconds and then in dioxane for 2 seconds. Finally, the dyed sections were transferred to xylene for 5 minutes, mounted with Canada balsam, and examined under a Leica light microscope.

Results and Discussion

Physical and chemical characteristics

The key physical and chemical characteristics of J5 brown dye observed during preparation, along with its stability under typical conditions, are summarized in Table 1. J5 brown dye was isolated as a brown powder with a yield of 87% and a melting point of 162–164°C. The infra-red (IR) spectrum (KBr, cm^{-1}) showed the following peaks: ν 3267 (OH), 3410 and 3332 (N-H), 1687 (C=O), 1597 (C=C aromatic), 1460 (N=N Azo), 1396 (C-N), and 1290 (C-O). The ^{1}H-NMR (DMSO, δ/ppm) exhibited the following signals: 7.72 (J = 7.73, 1.6 Hz), 7.56–7.36 (aromatic H), 6.84 and 6.37 (J = 2.4 Hz). The mass spectrum (ESI) showed a molecular ion peak at m/z = 229.24 $[M]^+$.[15] Spectral data are presented in Figures 1-3.

Table 1: Chemical and physical properties of the J5 brown dye

Compound	Molecular Formula	Molecular Weight(g.mol^{-1})	Melting Point ℃	Yield %	Color
J5 brown	$C_{12}H_{11}N_3O_2$	229.24	162-164	87	Brown

Effect of pH on acid-base behavior

A range of pH solutions, from 2 to 12, were tested to examine their effect on J5 brown dye. The ionization and protonization constants were determined by measuring absorption between 330 and 470 nm. The proposed chemical mechanisms for protonation and ionization are illustrated in Scheme 2. Figure 4 shows the absorption spectrum of the dye at various pH values. J5 brown exhibited isopiestic points,[16] at 330 nm, 380 nm, and 400 nm. Its maximum absorption occurred at 430 nm when the pH was between 2 and 8, and at 350 nm when the pH was between 9 and 12. Figure 5 illustrates the pH absorption curves for J5 brown dye, providing insight into its ionization and protonation constants. These constants were calculated and are presented in Table 2. The absorbance-pH curve was analyzed using the half-height method to determine the pK values, based on the following relation:[17]

$$pK = pH\ (at\ A½) \ldots\ldots\ldots\ldots (1)$$

$$A_{1/2} = (A_L + A_{min})/2 \ldots\ldots (2)$$

Where A_L and A_{min} represent the limiting and minimum absorbance, respectively.

Effect of solvent polarity on the Jamudiazene Brown dye

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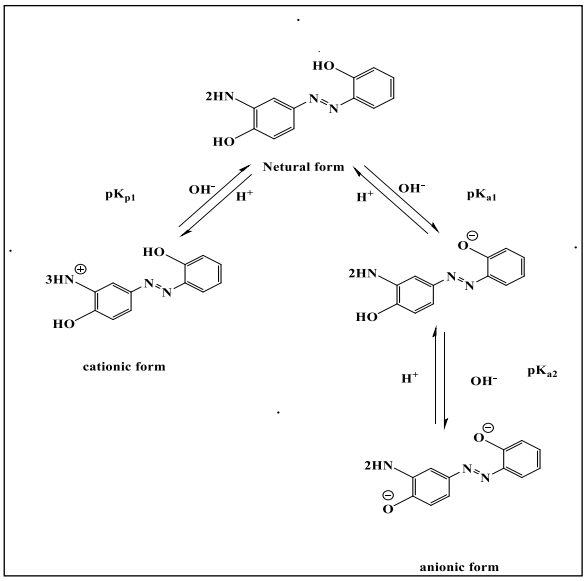
Figure 6 presents the spectra of J5 brown dye tested in various solvents with differing polarities, including n-hexane, 1,4-dioxane, chloroform, acetone, ethanol, methanol, DMSO, and water.[18]



Scheme 2: Mechanism of the J5 brown dye.

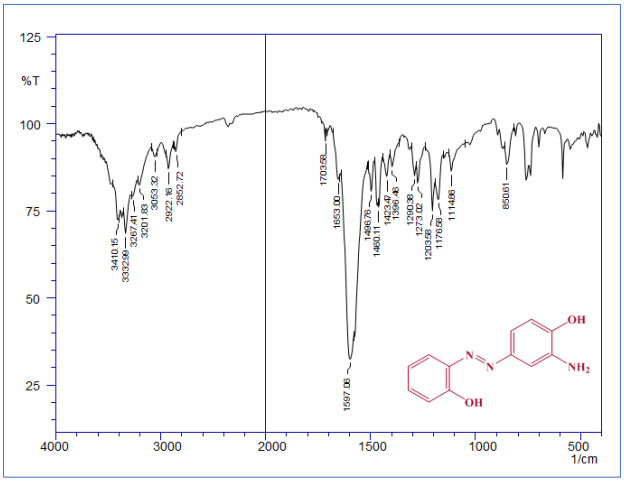


Figure 1: Infra-red spectra of the J5 brown dye.

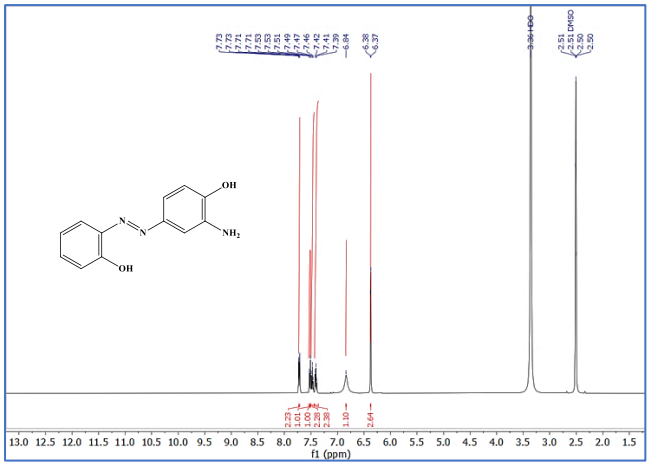


Figure 2: Proton nuclear magnetic resonance (^{1}HNMR) spectra of the J5 brown dye.

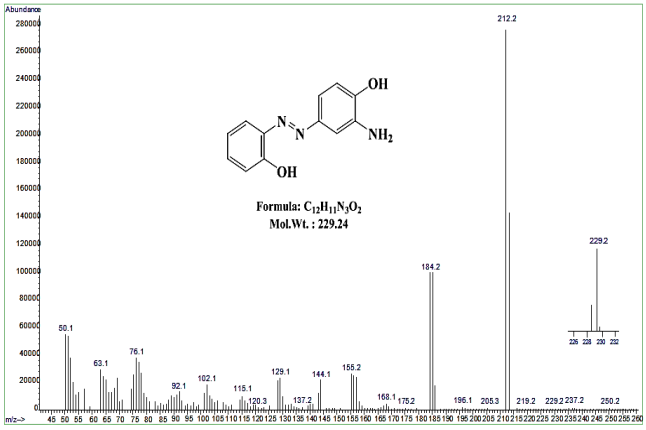


Figure 3: Mass spectra of the J5 brown dye.

The longest wavelength observed was 390 nm, with measurements taken to evaluate protonation and ionization effects. As shown in Figure 5, a red shift was noted in the spectra for the following solvents: n-hexane (400 nm), DMSO (490 nm), water (420 nm), acetone (420 nm), and chloroform (410 nm). No blue shift was observed. The J5 brown dye exhibited distinct spectra in various polar solvents, as summarized in Tables 3 and 4. The maximum wavelength, along with the observed redshifts and blue shifts, was identified.

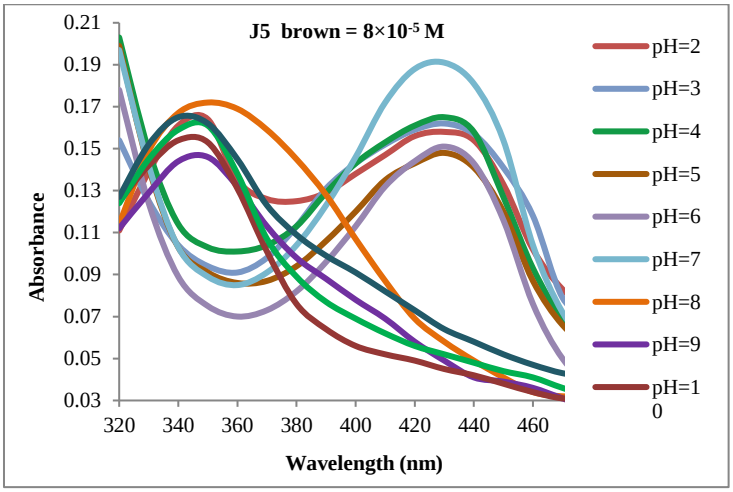


Figure 4: Absorption spectra at pH levels of 2-12.

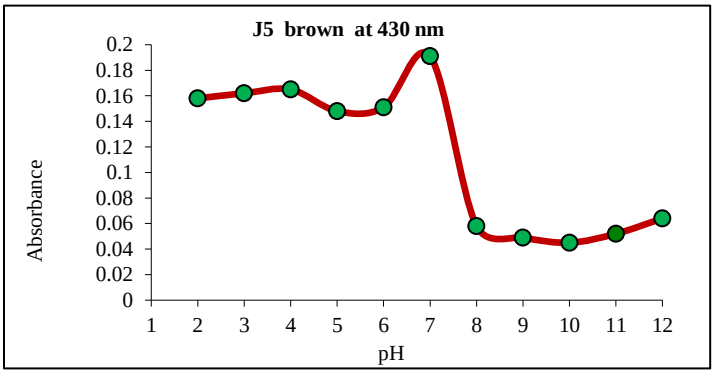


Figure: 5 The absorbance-pH curve of the J5 brown dye

Additionally, the wavelengths and molar absorption coefficients of J5 brown in these polar solvents were determined. According to the results in Table 5, there is a linear relationship between the solvent's absorption peak and its dielectric constant, expressed as:

$(D-1/D+1)$ [19] ……(3)

Table 2: Ionization and protonization constants of the J5 brown dye

J5 brown at λ= (430)nm			
A_m	A_L	$A_{½}$	pK
0.158	0.165	0.16	3 $_p$
0.148	0.191	0.169	6.51 $_{a1}$
0.045	0.064	0.054	>11 $_{a2}$

p = H's protonization constant for the phenol molecule's nitrogen atom.
a = H's ionization constant for the hydroxyl group in the phenol molecule.

Table 3: Information, Blue Shift and Red Shift

Compound	λ_{max} to 1,4-Dioxane	λ_{max}	Blue shift	Red shift	Notes shift
J5 brown	390	390	---------	n-Hexane ,Chloroform, Acetone and Water DMSO	weak shift medium Strong shift

Table 4: Information obtained from the use of different polar solvents

Solvent	n-Hexane	1,4-Dioxane	Chloroform	Acetone	Ethanol	Methanol	DMSO	Water
λ_{max}	400	390	410	420	390	390	490	420
$\mathcal{E}10^4$	0.176	0.628	0.363	0.242	0.432	0.472	0.441	0.251

This indicated that absorption is influenced by the solvent's dielectric constant, which according to the literature is represented by the (D) value.[20] The relationship between $(D-1)/(D+1)$ and the λ_{max} of the J5 brown dye was highly linear, as highlighted Figure 7. The absorption spectrum of J5 brown dye was measured in ethanol at a wavelength of 390 nm, with an absorption value of 0.346, as shown in Figure 8. The measurement was conducted using a 1 cm quartz cell. Ethanol served as the solvent to facilitate dye preparation and optimize the material for tissue staining. It also aided in refining the staining process after determining the dye's polarity, as illustrated in Table 5 and Figure 7.

Table 5: Effect of solvent on the spectra of the J5 brown dye

Solvent	symbol	D	(D-1)/(D+1)	λ_{max}
n-Hexane	1	1.89	0.308	400 $_W$
1,4-Dioxane	2	2.30	0.394	390
Chloroform	3	4.80	0.655	410 $_W$
Acetone	4	20.60	0.907	420 $_M$
Ethanol	5	24.00	0.920	390
Methanol	6	33.60	0.942	390
DMSO	7	46.67	0.958	490 $_S$
Water	8	78.30	0.975.	420 $_M$

Effect of J5 Brown dye staining on the histological components of fish tissue

The analysis of fish tissue samples stained with J5 brown revealed its effectiveness in colouring tissues, particularly bone structures. The bone sections appeared prominently brown, as shown in Figures 9-11. Compared to sections stained with Alizarin Red, illustrated in Figures 12-14, J5 brown demonstrated superior capability in in staining bone histological sections.[21] The chemical binding mechanism between the J5 brown dye structure and the components of animal histological sections can be explained by the potential interaction of negatively charged oxygen atoms in the dye with elements found in bone tissue.[22] Calcium is the most abundant component in bone tissue, with some calcium groups carrying positive charges that facilitate the bonding process, allowing the dye to associate with bone histology. After preparing a high-purity ethanol solution (99.9%) with a pH of 9 for the ionized dye, the binding strength of the dye to calcium-containing bone tissue increased, leading to enhanced mineralization of the bone tissue and clear colouration of bone histological sections.[23] The pigmentation process demonstrated approximately 80% accuracy and clarity compared to the pigmentation achieved with red Alizarin.[24]

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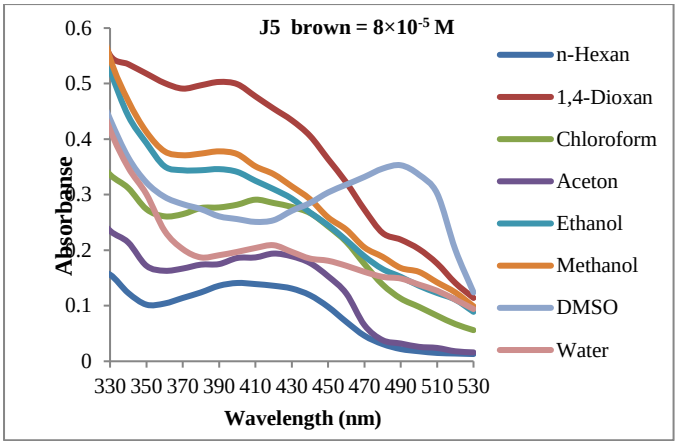

Figure 6: Electronic spectra of the J5 brown dye in different polar solvents.

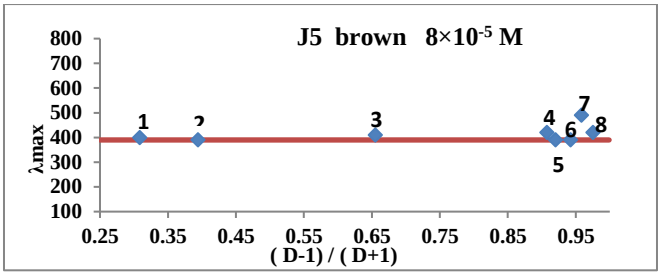

Figure 7: The relationship between (λ_{max}) and (D-1)/(D+1) for the J5 brown dye.

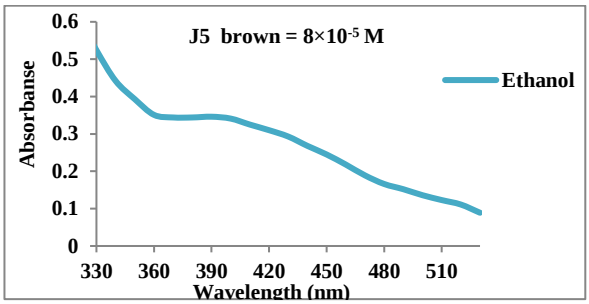

Figure 8: Spectral absorption for the J5 brown dye in ethanol.

J5 brown dye

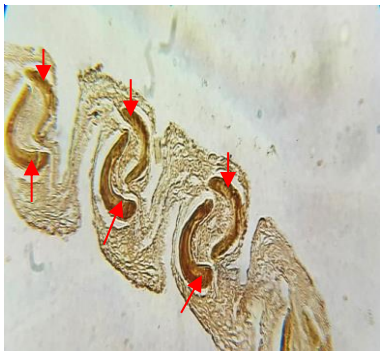

Figure 9: Cross-section in the fish's fin. Note the colouring of the fin bones with brown colour (arrows; 400x).

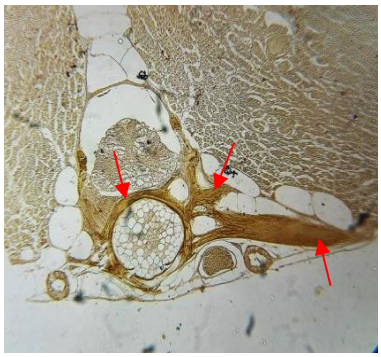

Figure 10: Cross-section in the trunk vertebrae of fish. Note the colouring of bone with brown colour (arrows; 100x).

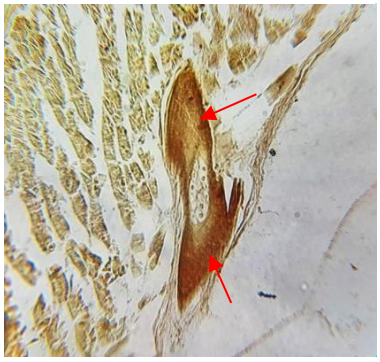

Figure 11: Cross-section in the rib area of the fish. Note the colouring of bone with brown colour (arrows; 100x).

alizarin red

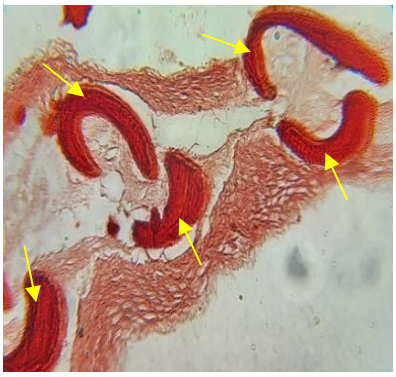

Figure 12: Cross-section in the fish's fin. Note the colouring of the fin bones with red colour (arrows; 400x).

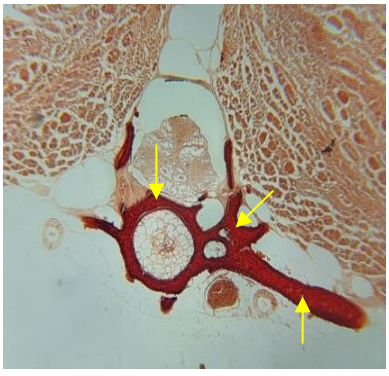

Figure 13: Cross-section in the trunk vertebrae of fish. Note the colouring of bone with red colour (arrows; 100x).

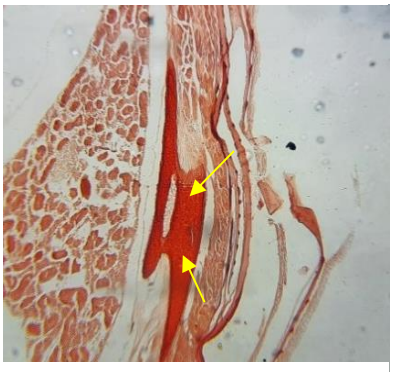

Figure 14: Cross-section in the rib area of the fish. Note the colouring of bone with red colour (arrows; 100x).

Conclusion

The synthesis of J5 brown dye was confirmed using the diagnostic techniques described in this study. The materials used for its preparation are low-cost, and the process is straightforward. J5 brown is effective in identifying bones and assessing their mineralization, functioning similarly to Alizarin Red for bone tissue identification. Additionally, the dye's high stability in solution ensures its long-term usability after preparation. The future direction of the study involves experimenting with different types of plant and animal tissues using J5 brown dye, as well as investigating its toxicity to assess its suitability for use in laboratory studies.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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