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Synthesis, Physicochemical and Microbial Evaluation of Phenylazo-1-Naphtholazobenzene (A Novel Disazo Dye)

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Abstract

Azo dyes are the most important synthetic colourants which have been widely used in textile, paper manufacturing and printing. A novel disazo dye, Phenylazo-1-naphtholazobenzene was synthesized and characterized using UV-Visible, FTIR spectrophotometric and elemental analytical tools. The result of the elemental analysis was found to be in conformity with the molecular formular of the dye. The UV-Visible spectra indicated that the dye absorbed at visible region of 499.0 nm, characteristics of intensely coloured dyes. The FTIR spectral study showed absorption bands conforming to the various functional groups: - OH stretch of non-bounded hydrogen at 3169.38cm^{-1}, CH stretch of alkenes and aromatics at 3015.26 cm^{-1}. C=C stretch of a conjugated aromatic compounds at 1654.15 cm^{-1}, -N^{+}=N of stretch of diazonium salt at 2277.13cm^{-1}, - N=N- group stretching at 1452.02 cm^{-1}, C-OH out of plane deformation at 6070.60cm^{-1} and NH deformation at 721.62 cm^{-1} in the dye. The colour imparted by the dye on the tested fabrics using Gray Scale for colour change, indicated very good to excellent lightfastness, and heatfastness, and very fair to good washfastness, except polyester and fine worsted wool which showed fair washfastness. The toxicological testing indicated that the dye has no effect on the Deoxyribonucleic Acid (DNA) of *Escheriachia coli* and *Aspergillus niger* but at high concentration, it denatured human DNA. The dye did not have adverse effect on the growth of *Zea mays* used for growth profile evaluation. Microbial toxicity of the synthesized dye on *E. coli, A. niger* and *Bacillus cereus* showed that all the strains are moderately resistant to the dye. The synthesized dye showed very good to excellent colourfastness, and fibres, not safe for human consumption, however, they are not toxic to the environment.

Key Words: Colourfastness, dye, environment, fibre, microorganism, toxicology.

Introduction

Across the world, in different industries like textile, leather, paper, food, *etcetera*, commercial dyes find huge applications. Synthetic organic colourants are often made from petroleum compounds which are carbon based. Most of the synthetic pigments except carbon black are not stable and wear away at the time of application [1]. Synthesis of dyes had

witnessed increase in a bid to obtain dyes with better fastness properties. Modern colourants are synthetic in nature, and majority of the commercial dyes belong to azo dyes. Depending on the chemical features, these dyes fall into several categories defined by the features for which they have affinity or by the methods by which they are applied [2].

Several researchers have synthesized disperse dyes with modified molecular structures aimed at enhancing properties like colour fastness, intensity and strength on polyesters. These new disperse dyes include dyes with long conjugation of naphthalene ring [3], pyrazole-based dyes [4]. The growing demand for dyes of high fastness ability, effective and of low environmental impact which would ensure that a synthesized dye achieves its intended goal gave rise to this study.

Therefore, this study reports the synthesis, physicochemical and microbial evaluation of Phenylazo-1-Naphtholazobenzene (A novel Disazodye).

Materials and Methods

Materials

All the reagents used were of the purest grades and were used as supplied unless stated otherwise. The microorganisms were isolates from Department of Microbiology, Chukwuemeka Odumegwu Ojukwu University, Uli Campus, Anambra State, Nigeria.

Methods

Synthesis of Phenylazo-1-naphtholazobenzene

Preparation of 2-naphthol solution

Solution of 1-naphthol was prepared by dissolving 14.4 g of the compound in 150.0 cm^3 of 2 M solution of sodium hydroxide.

Preparation of sodium nitrite solution

A 50 cm^3 of 2 M solution of sodium nitrite was prepared by dissolving 6.9 g of sodium nitrite in 50 cm^3 of distilled water.

First diazotization

A 22.0 cm^3 of concentrated hydrochloric acid was mixed with 100.0 cm^3 of distilled water. The solution was used to dissolve 9.3 g of aniline and the solution formed was cooled to 0 °C. Cold 50.0 cm^3 of the prepared sodium nitrite was slowly added with stirring for 10 minutes, maintaining the temperature at 0 °C. Thereafter the solution of the prepared 1-naphthol was coupled with the diazonium salt solution (prepared previously) by mixing the solution slowly with stirring for 30 minutes. A maroon dye was obtained, filtered, washed with distilled water, dried and then recrystalized in acetone.

Second diazotization

The second diazotization was done by dissolving 2.3 g of aniline in a mixture of 100.0 cm^3 water and 22.0 cm^3 concentrated hydrochloric acid and the solution cooled to 0 °C. The 50 cm^3 of cold 2 M sodium nitrite solution prepared by dissolving 6.9 g

of sodium nitrite in 50 cm^3 of distilled water was slowly added with stirring for 10 minutes, maintaining the temperature at 0 °C. Sodium hydroxide solution prepared by dissolving 1.5 g of sodium hydroxide pellets in 38 cm^3 of distilled water was used to dissolve 6.0 g of the monoazo dye prepared previously.

This was coupled to the benzene diazonium salt solution over 30 minutes with continuous stirring. The red dye obtained was filtered with Whatman No. 11cm filter paper, washed with distilled water, and dried in free air for 24 hours.

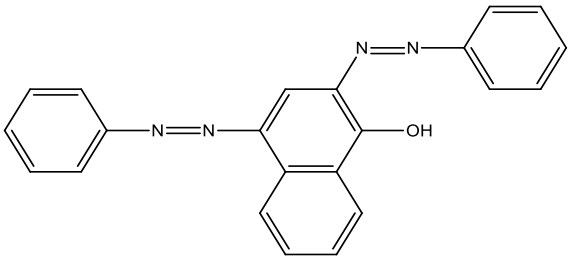

Fig 1: Phenylazo-1-naphtholazobenzene

Chromatographic Analysisand Percentage Yield Determination

The purity of the prepared dye sample was checked by Thin Layer Chromatography on a rectangular silica gel TLC plate (3.5 cm x 10 cm) using 3-methoxy ethane/water/acetone solvent mixture (5:2:3 ratio) as the mobile solvent, and the retardation factor (R_f) value was calculated by the mathematical expression,

$$R_f = \frac{Distance\ moved\ by\ the\ sample}{Distance\ moved\ by\ the solvent}$$

The percentage yield was determined using the relationship

$$\%\ \text{yield} = \frac{MR}{MMR} \div \frac{MP}{MMP} \times \frac{100}{1}$$

Where MR = mass of the reactant

MMR = molar mass of the reactant

MP = mass of the product

MMP = molar mass of the product

Physical Measurement

Melting point of the dye was determined using Perkin Elmar DSC 8500 (United Kindom) melting point apparatus. The elements present were detected using Thermo Jarrel Ash IRIS Inductively Coupled Plasma Optical Emission Spectrometer, (United States of America)

UV-Visible absorption spectra of the dye scanned from 900nm to 200nm using 10.0 cm^3 methanol was obtained on Jenway model 6850 spectrophotometer (United Kindom). Infrared (IR) spectra of the compound was recorded on a Perkin Elmer 100FTIR spectrophotometer (United Kindom).

Dying of fabrics

The pieces of Doctor Flannel worsted wool, fine worsted wool, 100% cotton, polyester, acrylic fibre and nylon, (each 2x3 cm) were respectively immersed in methanol-dye solution contained in a clean 250 cm^3 beaker and continuously stirred gently with a glass rod for 50 minutes. It was thereafter allowed to stand for 24 hours with occasional swirling of the beaker. Then, the pieces of dyed fabrics were carefully removed from the beaker, rinsed with enough distilled water, placed

on a white cardboard paper and allowed to dry in a free air for 24 hours [5, 6].

Heat fastness Evaluation

Heatfastness evaluation was carried out using the method described by Ukponmwen *et al.* [7]. The dyed fabrics were placed on a white background flat surface. They were heat-pressed with an electric pressing iron at a medium temperature of 60 °C for 30 seconds.

Lightfastness Evaluation

Lightfastness evaluation was done using the method described by Odilora *et al.* [8]. The dyed fabrics were firmly affixed to a white cardboard paper with office pin and exposed to hot sunlight for 2 hours. Thereafter, the exposed dyed fabrics were compared with the unexposed fabrics (control samples), to see if any colour change has occurred.

Washfastness Evaluation

Washfastness test was conducted as described by Odilora and Ogaga [9]. "Good Mama" detergent solution was prepared by dissolving 0.5 g of the detergent in 30cm^3 of distilled water in a 250 cm^3 beakers at a temperature of 45 °C separately. The dyed fabrics were immersed in the solutions respectively and vigorously stirred continuously for 5 minutes with a glass rod. The washed fabrics were then carefully removed from the detergent solution, rinsed with sufficient water, placed in a white cardboard paper and left to dry in free air for 24 hours.

Gray Scale Assessment

Analysis of the results of the fastness tests was done using the American Association of Textile Chemists and Colorists (A.A.T.C.C) Gray Scale for Colour Change. The fastness-tested dyed fabrics were compared with the control samples (corresponding to the untested dyed fabrics) to determine if any colour change(s) had occurred and to what extent if it did occur. The Gray Scale is graded 1-5 where 1 stands for very poor result and 5 for excellent result [10, 7].

Microbial Evaluation

The microbial evaluation was done by genotoxicity testing, seed germination bioassay and beneficial bacterial toxicity.

Genomic DNA extraction

Genomic DNA of *Escherichia coli* and *Aspergillus niger* and human blood were extracted using Quick-DNA™ Fungal/Bacterial Miniprep Kit according to recommended protocol (Zymo Research).

About 50-100 mg (wet weight) pure fungi cells that had resuspended in up to 200 µL of water was added to a ZR bashing bead lysis tube (0.1 mm and 0.5 mm). Seven hundred and fifty (750 µL) bashing bead TM buffer was added to the tube and vortexed at maximum speed for ≥ 15 min. ZR Bashing Bead™ Lysis tube (0.1 and 0.5 mm) was centrifuged in a microcentrifuge at 10,000 g for 1min. Thereafter, 400µL of the supernatant liquid was added to a zymo-Spin™ III-F filter in a collection tube and centrifuged at 8000 g for 1 min.

About 1,200 µL of genomic lysis buffer was added to the filtrate in the collection tube after which 800µL of the mixture was transferred to a zymo-spin™ IIC column in a collection tube and centrifuged at 10,000 g for 1min. The flow through was discarded from the collection tube and the step repeated. About 200 µL DNA pre-washed buffer was added to the Zymo-Spin™ IIC column in a new collection tube and centrifuged at 10,000 x g for 1 min. About 500 µL DNA wash buffer was as well added to the zymo-spinTM IIC column and centrifuged at 10,000 g for 1 min. The zymo-Spin™ IIC column was then transferred to a clean 1.5 mL microcentrifuge tube and 60 µL DNA elution buffer was added directly to the column matrix. Centrifugation was finally done at 10,000 g for 30 sec to elute the DNA.

Protocol for Bacterial Extraction

Genomic DNA was extracted using Quick-DNA™ Miniprep Plus Kit (Zymo Research), according to recommended protocol.

Each sample 200 µl was added to a microcentrifuge tube. BioFluid and Cell Buffer (200 µl) and 20 µl of proteinase K were added to it and mixed thoroughly using a vortex for 10-15 seconds and the tube containing the mixture incubated at 55 °C for ten minutes on a heating block. One volume Genomic Binding Buffer (i.e. 420 µl) was added to the digested sample and mixed thoroughly with a vortex mixer for 10-15 seconds. The mixture was then transferred to a Zymo-spin™ IIC-XL Column in a new Collection Tube and centrifuged at 12000 g for 1 minute. The collection tube was discarded with the flow through. DNA Pre-Wash Buffer (400 µl) was added to the spin column in a new Collection Tube and centrifuged at 12,000 g for 1 minute. The spin column was then transferred to clean microcentrifuge tube. DNA Elution Buffer (50 µl) was added directly on the matrix and incubated for 5 minutes at room temperature, and then centrifuged at maximum speed for 1 minute to elute the DNA. The eluted DNA was stored at ≤ -20 °C for future use.

Human DNA Extraction

Genomic DNA was extracted using QIAamp® Blood Mini Kit (QIAGEN), according to recommended protocol.

Protocol

QIAGEN Proteinase (20 µl) was added into a 1.5 cm^3 microcentrifuge tube. The sample (200 µl) and 200 µl of Buffer AL were added to it and mixed thoroughly using a votex for 10-15 seconds and then incubated the tube at 56 °C for 10 minutes on a heating block. The 1.5 cm^3 microcentrifuge tube was centrifuged to remove drops of the mixture from the lid. Ethanol (96-100%, 200 µl) was added and mixed thoroughly by votex. Tube was also centrifuged to remove drops from the lid. The mixture was then transferred to a QIAamp Mini spin Column in a 2 cm^3 Collection Tube and centrifuged at 6000 x g(8000 rpm) for 1 minute.

The collection tube was discarded with the flow. Buffer AW2 500 µl was added to the spin column in a new Collection Tube and centrifuged at full

speed for 1 minute (This eliminates the chance of possible Buffer AW2 carryover). The spin column was then transferred to a clean 1.5 cm^3 mocro-centrifuge. Buffer AE(200 µl)was added directly on the matrix and incubated for 1 minute below room temperature (15-25 °C), then centrifuged at 6,000 x g(8000 rpm) for 1minute to elute the DNA. The eluted DNA was stored at ≤ -20 °C for future use.

DNA Treatment with Synthesized Dye

The aliquots of the chemically synthesized dye at different concentrations (25 – 100 %) were added into the purified DNA sample, incubated at 37 °C for about 24 hours in order to determine the effect of the dye on the isolate.

Agarose gel electrophoresis

Two percent (2 %) agarose gel was prepared by dissolving 1.2 g of agarose in 60 mL of IX TAE buffer. The mixture was heated to a clear solution using a microwave oven and allowed to cool to about 50 °C. About 3 µL of ethidium bromide was added into the solution and mixed thoroughly. The agarose preparation was carefully poured into a gel tray with the gel comb in place and allowed to solidify. The tray was loaded into the gel tank and 1X TAE buffer was poured into the tank, making sure that the gel was properly submerged. The gel comb was carefully removed and 5 µL of exposed DNA was mixed with 2 µL of loading dye and loaded into the holes. The tank was connected to the power pack and set to run at 100 volts for 20 min. The bands were viewed using the gel documentation system (VILBER, GERMANY) [11].

Seed Germination Bioassay

The effect of synthesized dye at different concentrations was observed on *Zea mays* seed germination. The modified guideline for the testing of effluents on seeds of terrestrial plants according to the Organization for Economic Cooperation Development (OECD) [12] was used for this study. First, floatation method was adopted to determine the viability of the seeds. The seeds that sink represent the viable seed while the seeds that float represent seeds that are nonviable. Surface sterilization of the viable seeds was then performed by immersing them into 70 % ethanol for 5 minutes to reduce contamination and finally washed thoroughly with water. Ten maize seedlings were placed in Petri dishes lined with tissue paper containing 20 mL of the filtered dye at different concentrations (25 – 100 %) while the Petri dishes lined with tissue paper containing 20 mL of sterile distilled water served as the control. The investigation of each concentration including the control was carried out in triplicates.

The Petri dishes were covered with lids to prevent evaporation. The seeds were planted under room temperature for seven (7) days, after which the seed germination (%), shoot length (cm), root length (cm), relative root length percent and germination index (%) were measured and calculated [13]. The shoot length measured was taken from the base to the apical leaf of the plant using a transparent ruler;

while the root length was also measured by the same procedure after it was harvested and carefully washed with distilled water.

Beneficial Bacterial Toxicity

The effect of synthesized dye at different concentrations was observed on selected important bacterial strains' growth by measuring zones of inhibition using disc diffusion technique. In this method, nutrient broth and potato dextrose broth media were used to develop isolate *E. coli*, *Bacillus cereus*, and *Aspergillus niger*. After incubation for 48 hr, the surface of Muller Hinton agar plate was seeded with 48 hr grown *E. coli*, *Bacillus cereus*, and *Aspergillus niger* test organisms. Sterile filter paper discs impregnated with different concentrations of the dye (25 -100 %) and sterile distilled water as control were kept on the seeded bacterial cells at equidistance and pressed lightly and kept at 30 °C for 48 h; observation for zone of inhibition was made (if any) [14].

Results and Discussion

The synthesized dye has been investigated for its percentage yield, retardation factor, melting point, elemental composition, spectral analysis, fastness tests and microbial assay. The physicochemical data is recorded in Table 1.

Table 1: Physicochemical data of the dye

Colour	% yield	R_f value	Melting Point (°C)
Maroon	81.21	0.90	182

The coloured dye gave high percentage yield. Only one spot was observed on the thin layer chromatographic plate, showing that the synthesized dye was pure.

The melting point of the dye could be due to its intermolecular interactions which gave rise to strong cohesive bonds. Generally, as molecular weight increases, boiling point also increases.

Table 2 shows the elemental makeup of the dye.

Table 2: Elemental data of the dye

Carbon		**Nitrogen**		**Oxygen**		**Hydrogen**		**Total %**
No of atoms	%	No of atoms	%	No of atom	%	No of atom	%	
22	45.401	4	8.250	1	2.057	16	30.953	66.660

It is observed that the percentage composition of the elements in the dye compound is closely related to the theoretical values.

FTIR Spectroscopic Analysis of the dye

Table 3: FTIR Spectroscopic Analysis of the dye

Wave band cm^{-1}	Functional Group	Compound
670.60	C – OH out of plane deformation	Alcohol, phenol
721.64	CH out of plane deformation	Aromatic
971.80	Ring breathing mode	Cyclic
1119.24	C – O stretch	Tertiary alcohol
1149.03	C – X stretch	Aromatic halo
1293.73	Phenyl – N	Amino
1452.02	- N = N -	Azo
1510.44	C = C stretch	vinylidene
1703.31	C = C stretch	Aromatic
1903.21	Substituted benzene ring	Benzene
1992.87	Substituted benzene ring	Benzene
2174.07	C ≡ N stretch	Thiocyanate
2277.13	- $N^+ \equiv N$	Diazonium
2362.21	- $N^+ \equiv N$	Diazonium
2441.53	R – C = N stretch	Nitrile
2623.35	$R_3C = O$	Carbonyl
2792.11	CH stretch	Carbonyl
2865.02	-CH stretch	Trisubstituted
2930.47	CH stretch	Phenolic
3015.26	CH stretch	Aromatic
3169.38	RNH_2	1°amide
3243.13	NH stretch	Aromatic amine
3397.48	NH stretch	Aromatic amine
3470.72	NH stretch	Aromatic amine
3678.10	-OH	Alcohol, phenol
3759.50	-OH	Alcohol, phenol
3863.31	-OH	Alcohol, phenol
3946.68	-OH	Alcohol, phenol

Results in Table 3 supported the suggested structure of Dye 7 with the -OH stretch of non-bonded hydrogen bonds appearing at 3169.38 cm^{-1} to 3946.68 cm^{-1}. At 3015.26 cm^{-1} appeared the CH stretch of alkenes and aromatics whereas at 1654.15 cm^{-1} appeared the C =C stretch of a conjugated aromatic compounds. The – N^+= N stretch of diazonium salt was observed at 2277.13 cm^{-1} and azo group - N= N- stretching appeared at 1452.02 cm^{-1}. The C – OH out- of- plane deformation

appeared at 670.60 cm^{-1} and NH deformation at 721.62 cm^{-1}.

The UV-Visible Analysis Of the Dye

Table 4: UV-visible of the dye

Wave length(λ_{max})(nm)	**Bond**	**Transition**
218.0	C = C	$\pi \longrightarrow \pi^*$
300.0	C = C	$\pi \longrightarrow \pi^*$
357.0	C = C	$\pi \longrightarrow \pi^*$
499.0	C – OH	$n \longrightarrow \pi^*$

The two possible transitions are expected for the investigated Dyes 7 (the $\pi \rightarrow \pi^*$ and ($n \rightarrow \pi^*$) which however depends on the class of the chromophore and the nature of auxochrome. According to Bouas-Laurent and Durr, [15], Coelho *et al*. [16], Patil *et al*. [17], Scultz and Julius [18], the presence of OH group in the aromatic ring and two stable azo chromophoric groups (-N=N-) conjugated with aromatic rings resulted in molecules with intense and highly stable colourthroughout the visible region of the light. As shown in Table 4 the preliminary examination of the dye revealed, as expected two main bands observed at wavelength 218 – 357 nm were assigned to high energy $\pi \rightarrow \pi^*$ transition. The second band at 499 nm (visible region) was assigned to low energy $n \rightarrow \pi^*$ transition. According to Joseph et al. [19], the greater the distance between the ends of a conjugated chromophore, the greater is ε_{max}. So, increasing conjugation generally involve the absorption to a longer wavelength and finally into the visible region. Therefore, the prepared dyes having distinctive peak at 499 nm confirmed the dye to be conjugated and possess remarkable colour.

Fastness Evaluation

Table 5: Fastness evaluation data of the dye

Parameter	Fabrics					
	Dr Flannels worsted wool	Fine worsted wool	100% cotton	Polyester	Acrylic fibre	Nylon
Light fastness	5	5	5	4-5	5	5
Heat fastness	5	5	5	5	5	4.5
Wash fastness	3-4	3	4	3	4	4

Note: Grade 5=excellent, 4-5=very good, 4=good, 3-4=very fair, 3=fair, 2-3=poor, 1-2=very poor

As shown in Table 5, dyed sample exhibited acceptable lightfastness and heatfastness with rating ranging from 1-5 on the international Geometric Grey Scale[20, 21]. The dye showed excellent lightfastness and heatfastness on all the fibres except on polyester and nylon where it showed very good lightfastness and heatfastness respectively. This means that the materials retained their colour appearances longer, making it more durable and long-lasting. This observation is consistent with the notion that azo dyes containing electron withdrawing substituent on their diazo components exhibit reduced susceptibility to photofading [22].

One of the critical prerequisites for dyed fabrics is their Scorch fastness, which refers to their ability to withstand heat treatment. The synthesized dye demonstrated very good (4 - 5) to excellent (5) fastness to heat-pressing at 60°C and excellent photofading resistance.

The dye exhibited poor to very fair to good washfastness as measured by the international Geometric

DNA treated with the synthetic dye

It was observed that the dye did not denature the DNA of *E. coli* and *A. niger* after 24 hours. The same dye, at low concentration (12.5-25%) did not denature the human DNA. At higher concentration (50-100%), however, it denatured human DNA after 24 hours. According to Chung *et al* [23], the intake of azo dyes can increase the risk of human bladder cancer, splenic sarcomes and nuclear anomalis. Chung [24] also reported that azo dyes can cause allergic dermatitis, and even DNA damage that results in the formation of malignant tumors.

Effect of the dye on the growth of *Zea mays*

Table 6: Growth profile of *Zea mays* seeds exposed to different concentrations of the synthesized dye

Dilution	Dye Growth indices		
	No of germinated seeds	Root length (mm)	Shoot length (mm)
100%	5	18	9.0
50%	7	15	6.0
25%	7	18	16.0
12.5%	4	19	18.0
6.5%	4	11	16.0
Control (0%)	10	30	26.5

NB: Root length greater than 5mm and more than 5 germinated seed means no toxicity.

The result from Table 6 shows that the synthesized dye had no toxic effect on the growth of *Zea mays*. This was against the report according to Camen and Daniela [25], that the pollutants negatively affect the chlorophyll content of plants and they trigger the promotion of chlorophyllase and abscissic acid, both of which can lead to chlorophyll degradation.

Toxicity Assessment on Microbial Strains

The dye was tested for its possible toxicity on some microbes, and the result obtained from this aspect of study is presented in Table 7.

Table 7: Microbial toxicity of the synthesized dye

Dilution	Dye Zone of inhibition		
	E.coli	*Bacillus cereus*	*Aspergillus niger*
1000 mg/L	10	0	9
500 mg/L	9	0	0
250 mg/L	10	8	9
125 mg/L	9	8	8
65.5 mg/L	10	8	8
Control (0 mg/L)	0	0	0

NB: 0-9 = resistant, 10-19 = Intermediate or moderately resistant, 20-above = sensitive or susceptible to the dye.

All the organisms showed resistant to the dye with *E. coli* having moderate resistances at the1000 mg/L, 250 mg/L and 65.5mg/L dilution than other microbial strains.

Conclusion

A novel disazo dye, 4-chlorophenylazo-2-naphtholazo-4-chlorobenzene have been synthesized by stepwise diazotization and coupling reactions. The results showed that disazo dyes can be obtained in a high yield from aniline and 4-substituted anilines. The dye obtained showed very good to excellent colourfastness on all the fibres except washfastness where the dye performed poorly on almost all the fibres. The synthesized dye is not safe for human consumption and cannot be recommended for food and drug colourings. However, the dye is not toxic to plants and environment.

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