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Synthesis and Biological Evaluation of Isocyano-Functionalized Azo Dyes as Potential Antimicrobial Agents

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ABSTRACT
The study presents the synthesis, application and antimicrobial properties of azo dyes derived from substituted Isocyano-Functionalized Azo Dyes as diazo component with Gamma and H-acid as coupling components to produce Dye 1 and Dye 2 respectively. The physical characterization revealed yields of 70% and 80%, with melting points of 216–218 °C for Dye 1 and 195–197 °C for Dye2. UV–visible spectroscopic analysis showed absorption maxima at 473 nm for Dye 1 and 488 nm for Dye 2, confirming strong $\pi \rightarrow \pi*$ transitions and extended conjugation. FT–IR spectra reveals the presence of functional groups such as hydroxyl, amino, nitro, and sulfonate groups, acting as auxochromes that enhance the colour, solubility and stability of the dye products. The dyeing performance on silk and cotton fabrics demonstrated excellent affinity, with wash fastness ratings of 4 and 5, and light fastness ratings of 5 and 6 for Dye 1 and Dye 2 respectively. Dye 2 consistently exhibited superior fastness, attributed to stronger dye–fiber interactions and enhanced photostability. antimicrobial assays revealed broad-spectrum activity against staphylococcus aureus, escherichia coli, candida albicans, and aspergillus niger. The zones of inhibition showed Dye 2 to be more potent than Dye 1, particularly against S. aureus (33 mm vs. 25 mm) and C. albicans (28 mm vs. 21 mm). The MIC values confirmed strong inhibitory potential, with dye 2 exhibiting lower values against fungal isolates (12.5 mg/ml) compared to dye 1. The obtained MBC/MFC results further supported these findings, with dye 2 showing superior bactericidal/fungicidal activity.

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INTRODUCTION

Dyes are substances used to impart color to textiles, paper, leather, and other materials such that the coloring is not readily altered by washing, heat, light, or other factors to which the material is likely to be exposed. Dyes differ from pigments, which are finely ground solids dispersed in a liquid, such as paint or ink, or blended with other materials. Most dyes are organic compounds (i.e., they contain carbon), whereas pigments may be inorganic compounds (i.e., they do not contain carbon) or organic compounds. Pigments generally give brighter colors and may be dyes that are insoluble in the medium employed. Color has always fascinated humankind, for both aesthetic and social reasons.

Throughout history, dyes and pigments have been major articles of commerce. Manufacture of virtually all commercial products involves color at some stage, and today some 9,000 colorants with more than 50,000 trade names are used. The large number is a consequence of the range of tints and hues desired, the chemical nature of the materials to be colored, and the fact that color is directly related to the molecular structure of the dye [1].

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The color of a dye is due to the presence of chromophoric groups. Chromophores are responsible for dye color due to their N-saturation, while auxochromes are responsible for dye–fiber reactions. Dyes are widely used to color substrates such as textile fibers, paper, leather, hair, fur, plastics, wax, cosmetics, and foodstuffs [2]. Based on the chemical structure of chromophores, there are 20–30 different groups of dyes. Azo (monoazo, diazo, triazo, polyazo), anthraquinone, phthalocyanine, and triarylmethane dyes are the most important groups [3].

The majority of industrially important azo dyes belong to the following classes: acid dyes, basic dyes, direct dyes, disperse dyes, mordant dyes, reactive dyes, and solvent dyes. Basic, direct, and reactive azo dyes are atomic dyes [4]. Dyes contain at least one nitrogen–nitrogen (N=N) double bond; however, many different structures exist. For example, in azo dyes, monoazo dyes have only one N=N double bond [5]. The azo groups are generally connected to benzene and naphthalene rings, with varying intensities possible [6]. These dyes have different absorption spectra associated with electronic transitions between molecular orbitals. Synthetic dyes are produced annually worldwide, but during the dyeing process, a substantial amount of azo dye is lost in waste [7]. A dye is a colored compound, normally used in solution, which is capable of being fixed to a fabric. The dye must be chemically stable so that color is not washed out with soap and water or faded by sunlight. Dyeing is normally done in a special solution containing dyes and particular chemical materials. After dyeing, dye molecules form chemical bonds with fiber molecules [8].

Colors of dyes are due to electronic transitions between various orbitals. The probability of these transitions determines the intensity of the color, while the energy difference between orbitals determines whether the color falls within the visible region of the electromagnetic spectrum (400–800 nm) [9]. Synthetic dyes offer several advantages over natural dyes, including a wider range of colors, superior color fastness, consistent quality, and cost-effectiveness. They are also more versatile, suitable for various materials and production scales. While natural dyes have eco-friendly and health benefits, synthetic dyes remain the preferred choice for many industries due to their ease of use and performance characteristics.

There is a growing need for dyes that not only provide color but also possess functional properties such as antimicrobial activity. While azo dyes are widely used for their vivid color and stability, their biological activity is often limited. 2-amino-4-isocyano-5-methyltetrahydrothiophene, a heterocyclic compound with reactive functional groups, offers potential as a novel precursor for synthesizing azo acid dyes with enhanced antimicrobial properties. However, little research has been done in this area. This study aims to fill that gap by synthesizing such dyes, evaluating their dyeing performance, and investigating their antimicrobial activity, especially for applications in medical or hygienic textiles [10].

This study is justified by the need to develop dyes that offer both coloration and antimicrobial properties, addressing current challenges in textile and material science. 2-amino-4-isocyano-5-methyltetrahydrothiophene is a novel heterocyclic compound that holds potential for producing azo acid dyes with enhanced functionality. Given the limited research on dyes derived from this compound, especially regarding their biological activity, this work could lead to the development of multifunctional dyes suitable for medical and hygienic applications, making it both scientifically relevant and practically valuable [11].

The aim of this research is to synthesize Azo acid dyes using heterocyclic based diazo component and to study their application and their antimicrobial properties. The key objectives of this research work are:

1. To synthesize Azo acid dyes based on substituted 2-amino-4-isocyano-5-methyltetrahydrotiophene as diazo amine component, with coupling component such as 3-amino benzoic acid and 2,4-diamino benzoic acid.

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2. To characterized the dyes using spectroscopic techniques such as UV-visible, FTIR and melting point.
3. To apply and investigate the fastness properties of the synthesize dyes on polyester fiber.
4. To investigate the antimicrobial properties of the synthesize dyes against some select bacteria and fungi.

MATERIALS AND METHODOLOGY

Materials

Chemical/Reagents

Hydrochloric acid, sodium-hydroxide, dimethylsulfocide, sulphuric acid, benzaldehyde, ethanol, phenylhydrazine, acetone, acetoacetanilide, malononitrile, sulphur, 1-naphthaldehyde, acetic acid, copper sulphate, sodium nitrite, dimethylformamide and gamma acid, and H-acid.

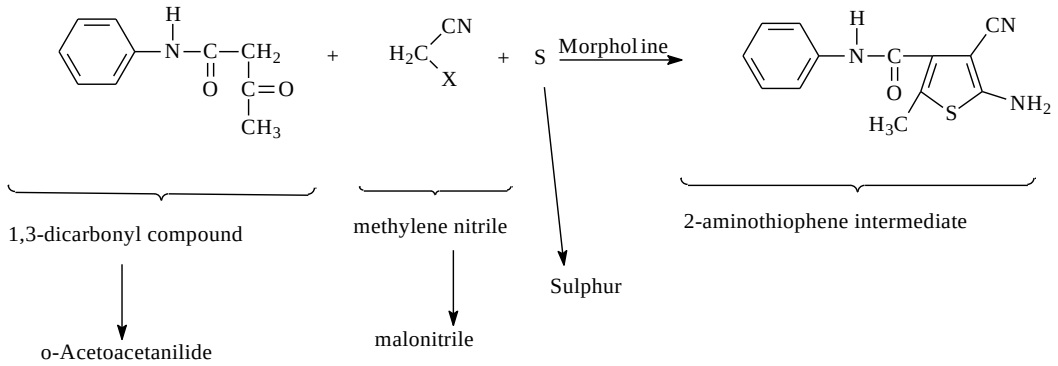
Apparatus and Equipment

Thermometer, pH meter, heating Mantle, Magnetic Stirrer, Electronic balance, Gallenkamp Melting Point apparatus, Agilent CARY 300 UV-Visible Spectrophotometer, Agilent CARY 630 FT-IR, Electric Oven, Beakers, round bottom flask, 250ml volumetric flasks, Buchner flask, hot plate, steam bath, sample bottles.

Methods

Synthesis of Aminothiophene Intermediate

The aminothiophene intermediate was synthesized following the Gewald's methods reported by [12]. O-acetoacetanilide (21.16 g, 0.1 mol), malononitrile (6.96 g, 0.1 mol) and sulphur (3.37 g, 0.1 mol) in 30 ml ethanol was stirred in the presence of morpholine (8.97g, 0.1mol) at 70 ^{o}C for 3 hours. The resulting thick dark solution was cooled and stored overnight in a refrigerator, followed by filtration, washing with ethanol and then ethanol/water (1:1) solution and dried. The powder was thereafter recrystallized from ethanol.



Diazotization of 2-Aminothiophere Intermediate and Coupling Reaction

Sodium nitrite (1.38g, 0.02mol) was added drop wise to 10 ml of concentrated sulphuric acid at 10^{o}C and heated to 60^{o}C with stirring for 15 minutes. The solution was cooled to 5^{o}C and a mixture of acetic acid and propionic acid (17:3) was added to the mixture below 30^{o}C. The finely ground aminothiophene intermediate (4.90 g, 0.02 mol) was added slowly within 30 minutes below 5^{o}C and the whole mixtures was stirred at 0-5^{o}C for 2hours.

The excess nitrous acid (which was tested for, using starch iodide paper) was decomposed with the required amount of urea. The clear diazonium salt solution thus obtained was used immediately in the coupling reaction. The associated steps are illustrated in the reaction scheme below.

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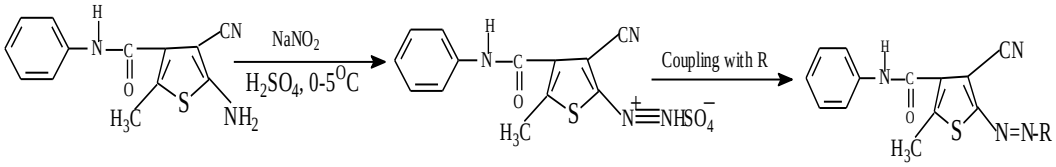
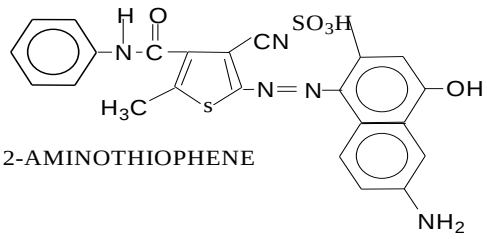


Where R represent coupling component (3-aminobenzoic acid and 3,4-diaminobenzoic acid)

Coupling Reaction

The coupling components (gamma acid and H-acid) were dissolved in 1 M sodium hydroxide solution and cooled by adding ice. The prepared solution of gamma acid and H-acid was then added to the diazonium salt solution of the aminothiophene intermediate over 30–40 minutes with vigorous stirring. The mixture was stirred for a further 30 minutes at 0–5 °C, after which the pH of the solution was adjusted to 5 by the addition of 10% sodium hydroxide solution. The resulting product was collected by filtration, and the crude product obtained was purified by recrystallization from ethanol to yield the respective dyes [12].

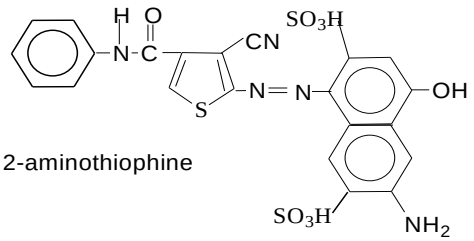
The proposed structures of the synthesized dyes are as follows:



2-AMINOTHIOPHENE

Gamma acid

DYE 1



2-aminothiophine

DYE 2 H-acid

Purification of the Intermediate

The aminothiophene derivatives were purified by three to four recrystallizations from ethanol. A known weight of the derivatives was dissolved in a small quantity of ethanol and heated with constant stirring. The solution was then filtered while cooling using a Buchner funnel with a suction pump. The crystals were collected, washed several times with water, and dried. After recrystallization, the purity of the heterocyclic amine was checked by spotting on a TLC plate using hexane:ethyl acetate (2:1) as the eluent [13].

Purification of Dye

The dyes were recrystallized by heating it in ethanol (60 °C). The solution was filtered out and then allowed to cool in an ice bath. The crystals formed was wash and dried in the oven at 50 °C and thereafter, the percentage yield was calculated.

Characterization of the Synthesized Dyes

The intermediate and the synthesized dyes were characterized via Fourier Transform Infra-Red Spectroscopy (FTIR) using Agilent CARY 630 FT-IR spectrophotometer; the melting point was determined by using Gallenkamp melting point apparatus and UV-Visible spectroscopy of the dyes were recorded on Agilent CARY 300 UV-Visible spectrophotometer in DMSO and methanol at a concentration of 1.0×10^{-5} mol/L, as described by Wang [14].

Application of Dyes

1% stock solution of each dye was prepared, a liquor ratio of 50:1 was used, 2% shade on weight of fabric (o.w.f) and 1g of the fabric. The volume required from each stock solution was calculated based on the formula below: $V = \frac{PXW}{C}$

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Where: $V = \frac{PXW}{C}$

P-represent the percentage shade
W- represent the weight of fabric
C-represent the percentage concentration of dye stock solution

Dyeing of Silk Fabric

The dyeing process was with the preparation of the dye bath. The pH of the dye bath was adjusted to the optimal range for silk dyeing, typically between 4.5 and 5.5 using acetic acid. The silk fiber was then added to the dye bath, and the mixture was heated to the desired temperature, usually around 80-90°C. The dyeing process was allowed to proceed for 1 hour, to achieve the desired shade and colour yield. After the dyeing process, the silk fabric was removed from the dye bath and rinse with cold water to remove excess dye. The fabric washed with a mild detergent and cold water and finally dry to evaluate the colour fastness and other dyeing characteristics.

Assessment of Fastness Properties

Wash Fastness Test

The dyed silk fabric was subjected to the ISO 3 wash fastness test using the following procedure: The specimens were prepared by cutting the dyed fabric into dimensions of 5 × 2 cm. They were thereafter made into composites by stitching the test specimen (dyed sample) placed in between white cotton fabric of dimensions 10 × 5 cm. The composites were then agitated in the prepared washing solution as described by Nkeonye [15].

Soap solution 5g/1
Sodium carbonate 2g/1
Liquor ratio 50:1

The wash was maintained at 50°C for 45 minutes with continuous agitation. At the end of the Wash test the composite specimen was remove, rinse in cold water and the components separated and dried at room temperature. The change in colour of the dyed fabric and the staining of adjacent undyed cloths was assess using the appropriate grey scale.

Light Fastness Test using 8-Blue Wool Standard

The dyed samples and Blue Wools Standard were expose facing due south and incline at an angle to the horizontal approximately equal to the latitude of the place where the exposure was made. Adequate ventilation of the samples during exposure was ensured. The partly covered sample was exposed to UV radiation. As exposure preceded for 14 days, the samples under test and the blue wool standards was examine at intervals and the change in colour of the samples was compared visually with the changes that occur in the standards. The light fastness of the sample was the number of the standard that shows a similar visual contrast between the expose and unexposed parts of the samples. Light fastness values for each material was obtained by comparing the degree of fading with that observed with the Blue Wool Standard. The rating was given according to the wool standard with which the dyed material fading is comparable [15].

Evaluation of the antimicrobial activities of Azo dye

Test Organisms

The organisms that were use for the analysis was clinical isolates of bacteria and fungi that was obtain from the department of microbiology. The isolates were: *Candida albicans, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae* and *Candida tropicalis*.

Culture media

The culture media that was use for the analysis include Mueller Hinton agar (MHA), Mueller Hinton Broth (MHB), potato Dextrose Agar (PDA) and Nutrient Agar (NA) These media was used for antimicrobial susceptibility testing Viz: zones of inhibition, Minimum inhibition concentration (MIC) and minimum Bactericidal/Fungicidal Concentration (MBC/MFC). All the media was prepared according to manufacturer's instruction and sterilized by autoclaving at 121°C for 15 minutes.

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Determination of zone of inhibition using Agar Well Diffusion Method.

The standard inoculate of both the bacterial and fungi isolates was streak on sterilized Mueller Hinton Agar and Potato Dextrose Agar plate with a sterilized cork borer. The well was properly labeled according to the different concentration of the synthesize dyes which is from 100, 50, 25, 12.5 and 6.5 mg/ml respectively. Each well was filled up with 0.2 ml of dye solution. The inoculated plate with the dye solution was allowed to stay on the bench for 1 hour, this is in order to allow the dye solution to diffuse on the agar. The plate of Mueller Hinton Agar was incubated at 37°C for 24 hours while the plate of Potato Dextrose Agar was incubated at room temperature (27°C) for 3 days. At the end of the incubation period, the plate was observed for any evidence of inhibition which was appear as a clear zone that was completely devoid of growth around the wells (zone of inhibition) the diameter of the zone was measured using a ruler calibrated in millimeter.

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations of the synthesized dyes were determined using the plate method with the Nutrients Agar and Potato Dextrose Agar. This is the lowest concentration of the synthesized dyes showing inhibition for each organism. The organisms were inoculated into each plate containing the agar and the synthesized dyes. The inoculated plates were then incubated at 37 °C for 24 hours.

At the end of the incubation period, the plate was observed for the presence or absence of growth using turbidity as a criterion, the lowest concentration in the series without visible sign of growth (turbidity) was then considered to be the minimum concentration (MIC).

Determination of Minimum bactericidal/fungicidal concentration (MBC/MFC)

The result from the minimum inhibitory concentration (MIC) was used to determine the minimum bactericidal/fungicidal concentration (MBC/MFC) of the synthesized dyes. A sterilized wire loop was use to take some parts on the plate that did not show turbidity (clear) in the MIC test and a loopful was taken and streak on a sterile nutrient agar plate. The plates were incubated at 37 °C for 24 hours.

At the end of incubation period, the plate was examined for the present or absent of growth. This is to determine whether the antimicrobial effect of the synthesized dye is bacteriostatic/fungistatic or bactericidal/fungicidal.

RESULTS AND DISCUSSION

Table 1: Physical Properties of the Synthesized dyes

Dye NO.	Molecular Formular	Molecular Weight (g/mol)	Colour of the Dye	% Yield	Melting point °C
D1	$C_{25}H_{17}O_4N_5S_2$	515.556	Marron	70	216-218
D2	$C_{25}H_{14}O_8N_5S_3$	608.59	Orange	80	195-197

Physical Properties of the Synthesized dyes

Table 1 presents the physical properties of the synthesized azo dyes (D1 and D2), including their molecular formulae, molecular weights, colors, percentage yields, and melting points. The data confirm successful synthesis of both dyes, as evidenced by their distinct coloration and melting points typical of stable aromatic azo compounds. The variation in color between the two dyes can be attributed to differences in their substituent groups and molecular conjugation. Specifically, D2 contains additional sulfonic acid and hydroxyl substituents, which enhance electron-withdrawing and donating resonance effects, thereby altering the chromophore's absorption characteristics and producing a visible spectral shift [16,17].

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The relatively high melting points observed for both dyes suggest strong intermolecular hydrogen bonding and high thermal stability, typical of aromatic azo dyes containing sulfonic and hydroxyl substituents. Such structural features contribute to rigid planar configurations with extensive conjugation, which in turn enhance stability and color intensity [18,19].

The percentage yields (70% for D1 and 80% for D2) are within the expected range for diazotization–coupling reactions. The slightly higher yield in D2 may be due to more efficient coupling facilitated by the electron-withdrawing sulfonic groups, which stabilize the diazonium intermediate and improve reaction efficiency [20]. These yields indicate successful reactions with minimal by-product formation. Overall, the results confirm that the synthesized dyes are thermally stable, intensely colored, and structurally consistent with azo-based chromophores derived from thiophene and naphthol derivatives. These findings are in agreement with previous reports on structurally similar azo dyes, which exhibit high color strength, good yields, and strong fastness properties [16,17].

Table 2: UV–Visible Spectral Interpretation of Dyes 1 and 2 in Ethanol

Dye Sample	λmax (nm)	Absorbance (A)	Type of Transition	Chromophore Involved	Interpretation/Observation
Dye 1	473	2.262	π → π* transition (main), possible weak n → π* shoulder	Conjugated C=C and –N=N (Azo or aromatic system)	Strong visible band indicates extended conjugation; broad band indicates overlapping π→π* and possible ICT effects.
Dye 2	488	0.326	π → π* transition with ICT contribution	Conjugated aromatic/azo system with donor–acceptor substituents	Slight bathochromic shift vs. Dye 1 suggests longer conjugation path or stronger electron-donating substituents.

Both dyes absorb in the visible region (470–490 nm), confirming extended π–conjugation typical of aromatic chromophores.

Table 3: FT–IR Spectral Interpretation and Functional Group Assignments

Dye	Observed Band (cm^{-1})	Intensity / Shape	Functional Group Assignment	Structural or Chemical Significance
Dye 1	3421–3278	Broad, strong	O–H / N–H stretching	Hydrogen-bonded hydroxyl or amino groups; auxochromes contributing to visible absorption.
Dye 1	2199	Medium	C≡N stretching (possible)	May indicate nitrile group; contributes to polarity and charge-transfer.
Dye 1	1650–1600	Strong	C=C (aromatic ring) / conjugated C=O	Confirms aromatic skeleton and π–conjugation.

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Dye	Observed Band (cm^{-1})	Intensity / Shape	Functional Group Assignment	Structural or Chemical Significance
Dye 1	1539–1458	Medium	$–NO_2$ asymmetric/symmetric stretches	Indicates nitro substitution; influences λmax shift.
Dye 1	1323–1020	Strong	C–N, C–O, S=O stretches	Suggests amine, ether, or sulfonate substituents enhancing solubility.
Dye 2	3652, 3318	Sharp and broad	Free and H-bonded O–H / N–H	Multiple hydroxyl/amine environments; donor sites for H-bonding.
Dye 2	2967–2922	Medium	C–H (aromatic/aliphatic)	Confirms substituted aromatic and/or alkyl groups.
Dye 2	1602–1491	Strong	C=C (aromatic), NO_2 asymmetric stretch	Confirms aromatic conjugation and nitro substituents.
Dye 2	1400–1200	Medium	C–O / C–N / S=O stretches	Polar auxochromic groups such as sulfonates or phenolics.

Table 4: Comparative Structural and Optical Summary

Property	Dye 1	Dye 2	Interpretation
λmax (nm)	473	488	Dye 2 red-shifted → increased conjugation.
Absorbance	High (2.26)	Low (0.33)	May arise from different concentration or molar absorptivity.
Chromophore Type	Conjugated aromatic / azo	Conjugated aromatic / azo	Both have similar chromophoric cores.
Auxochromes	–OH / $–NH_2$ / $–SO_3H$ / $–NO_2$	–OH / $–NH_2$ / $–SO_3H$ / $–NO_2$ (extra OH)	Auxochromes cause λmax shifts and solubility.
Structural Order	Planar, aromatic	Planar, extended conjugation	Dye 2 has greater delocalization.

UV-Visible Absorption and FT-IR Analysis

The UV–Vi's spectra of both dyes confirm the presence of strong π→π∗ electronic transitions typical of highly conjugated aromatic systems. Dye 1 exhibits an absorption maximum at 473 nm, whereas Dye 2 shows a slight bathochromic shift to 488 nm, indicating a higher degree of conjugation or enhanced donor–acceptor interactions. The FT–IR spectra further validate the presence of aromatic rings, hydroxyl, amino, nitro, and sulfonate groups that act as auxochromes, influencing electronic transitions and solubility. These functional groups are responsible for the characteristic visible absorption bands and the stability of the dyes in ethanol. The correlation between UV–Vis and FT–IR data reveals that both dyes are aromatic, planar, and contain polar substituents that extend conjugation and improve chromophoric performance [21].

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Table 5: Wash Fastness

S/N	SAMPLES	CHANGE IN COLOUR	STAINING OF ADJACENT FABRIC
1	Dye 1	4	4/5
2	Dye 2	5	4/5

Wash Fastness Properties of the Synthesized Dyes

Table 6 presents the wash fastness properties of the synthesized dyes (H-acid and Gamma-acid), including their ratings for color change and staining of adjacent fabrics. The data indicate that both dyes exhibit good to excellent wash fastness, as evidenced by their high ratings on the Gray Scale for Color Change and Staining, which are internationally recognized standards for assessing textile fastness properties (ISO 105-C06; AATCC Test Method 61).

The H-acid dyed sample recorded a rating of 4 for color change, corresponding to good wash fastness with only slight fading after repeated laundering. In contrast, the Gamma-acid dyed sample achieved a rating of 5, representing excellent wash fastness with no perceptible change in color. This difference can be attributed to the stronger molecular affinity of Gamma-acid towards the fiber, resulting in more stable dye–fiber interactions and reduced dye loss during washing [22,23]. For staining of adjacent fabrics, both dyes scored 4/5, indicating very minimal staining and excellent resistance to dye bleeding. This performance suggests that the synthesized dyes possess strong substantivity and are less prone to migration during washing. The presence of sulfonic acid groups enhances water solubility while maintaining fiber affinity, thereby improving wash fastness [24].

Overall, the findings confirm that both dyes demonstrate high wash fastness, with Gamma-acid showing superior color retention compared to H-acid. These results are consistent with previous studies on structurally similar acid dyes, which emphasize the role of substituent groups and molecular conjugation in enhancing fastness properties [25,26].

Table 6: light fastness

S/N	**SAMPLE**	**CHANGE IN COLOUR ON COTTON FABRIC**
1	Dye 1	5
2	Dye 2	6

Light Fastness Properties of the Synthesized Dyes

Table 6 presents the light fastness properties of the synthesized dyes (H-acid and Gamma-acid), evaluated on cotton fabric using the Blue Wool Scale, which ranges from 1 (very poor) to 8 (excellent). The results indicate that both dyes exhibit high resistance to fading under light exposure, confirming their suitability for applications requiring prolonged outdoor or sunlight exposure. The H-acid dyed sample recorded a rating of 5, which corresponds to good light fastness, showing moderate resistance to photo-degradation with only slight fading after extended exposure. In comparison, the Gamma-acid dyed sample achieved a rating of 6, representing very good light fastness with stronger resistance to color change. This difference can be attributed to the molecular structure of Gamma-acid, which likely provides enhanced stability against photo-oxidative reactions.

Dyes with extended conjugation and electron-withdrawing substituents are known to resist photo-degradation more effectively, thereby maintaining color intensity for longer periods [27,28]. The relatively high ratings observed for both dyes suggest that they possess strong chromophoric stability and reduced susceptibility to ultraviolet radiation. Such performance is consistent with the behavior of acid dyes

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containing sulfonic and hydroxyl groups, which enhance dye–fiber bonding and reduce photo-fading [29]. Overall, the results confirm that both dyes demonstrate good to very good light fastness, with Gamma-acid showing superior performance compared to H-acid. These findings align with previous studies on azo and acid dyes, which emphasize the importance of substituent groups and molecular resonance in improving photo-stability and fastness properties [30,31].

Table 7 Zone of Inhibition (mm) of the Test Organisms

Test Organism	Dye 1	Dye 2	Sparflo	Fluco
Staphylococcus aureus	25	33	37	-
Escherichia coli	28	29	35	-
Candida albicans	21	28	-	32
Aspergillus niger	18	21	-	35

Key: Sparflo: Sparfloxacin = 5µg/ml (Positive control drug for bacteria)
Fluco: Fluconazole = 5µg/ml (positive control drug for fungi)

The Zone of Inhibition (nm) of the Test Organisms

The antimicrobial activity assessment of the two natural dyes revealed that both exhibited inhibitory effects against the tested bacterial and fungal organisms, although with varying degrees of potency. Dye 2 consistently demonstrated stronger antimicrobial effects than Dye 1 across all isolates, indicating a higher concentration or better bioavailability of active compounds within its composition [32, 33].

For the bacterial isolates, *Staphylococcus aureus* and *Escherichia coli* showed distinct susceptibility patterns. *S. aureus* exhibited inhibition zones of 25 mm for Dye 1 and 33 mm for Dye 2, while *E. coli* recorded 28 mm and 29 mm, respectively. The greater sensitivity of *S. aureus* compared to *E. coli* is consistent with reports that Gram-positive bacteria are generally more susceptible to plant-derived compounds due to their simpler cell wall structure, which lacks the outer lipopolysaccharide layer characteristic of Gram-negative bacteria [34,35]. This structural difference facilitates better penetration of phytochemicals and dye molecules, leading to higher antimicrobial efficacy. When compared with the standard antibiotic Sparfloxacin (37 mm and 35 mm for *S. aureus* and *E. coli*, respectively), both dyes exhibited moderate antibacterial activity. Although less potent than the commercial drug, their inhibition zones indicate the presence of bioactive compounds capable of impeding bacterial growth [36].

The antifungal results further confirm the bioactivity of the dyes. Both *Candida albicans* and *Aspergillus niger* were inhibited by Dye 1 and Dye 2, with *C. albicans* showing inhibition zones of 21 mm and 28 mm, respectively, and *A. niger* 18 mm and 21 mm. Although these values were lower than those obtained for Fluconazole (32 mm and 35 mm), the results indicate moderate antifungal properties. The higher inhibition by Dye 2 suggests that its constituents, possibly polyphenolic or metal-complexed compounds, can disrupt fungal cell wall integrity or interfere with enzyme activity [37].

Overall, the antimicrobial profile suggests that both dyes, particularly Dye 2, have promising potential as synthetic antimicrobial agents. Their ability to inhibit both Gram-positive and Gram-negative bacteria, as well as fungal species, underscores their broad-spectrum activity. These findings are significant in the context of sustainable materials science, where synthetic heterocyclic dyes not only provide improved coloration but also impart functional properties such as antimicrobial protection [38]. Thus, the dual functionality of these dyes could be advantageous in applications such as textile finishing, leather treatment, or biomedical coatings, where heterocyclic dyes are preferred over conventional azo synthetic alternatives.

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Table 8 Minimum Inhibitory Concentration (MIC) in mg/ml

Test Organism	Dye 1	Dye 2
Staphylococcus Aureus	12.5	12.5
Escherichia Coli	25	50
Candida albicans	25	12.5
Aspergillus niger	25	25

The Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) data presented in Table provide insight into the antimicrobial potency of Dye 1 and Dye 2 against selected bacterial and fungal organisms. MIC values reflect the lowest concentration required to inhibit visible microbial growth and are therefore a critical parameter for assessing antimicrobial strength [39]. Lower MIC values denote higher antimicrobial efficacy. Both dyes exhibited considerable antimicrobial activity, though their performance varied across the tested organisms.

Against *Staphylococcus aureus*, both dyes produced identical MIC values of 12.5 mg/ml, demonstrating strong inhibitory potential toward this Gram-positive bacterium. This observation corresponds with the inhibition-zone results, where *S. aureus* also showed high susceptibility. The enhanced sensitivity of Gram-positive bacteria to plant-based or natural dye extracts has been widely reported and is attributed to the absence of an outer lipopolysaccharide membrane, allowing easier penetration of active phytochemicals [40,41]. For *Escherichia coli*, Dye 1 recorded a lower MIC value (25 mg/ml) than Dye 2 (50 mg/ml), suggesting relatively greater activity of Dye 1 against this Gram-negative strain. Gram-negative bacteria are generally more resistant to antimicrobial agents due to the protective outer membrane and efflux mechanisms that hinder the diffusion of many bioactive compounds [42,43].

The slight advantage of Dye 1 may therefore arise from specific chemical constituents such as smaller or more hydrophilic molecules that enhance its ability to cross bacterial membranes.

In the case of the fungal isolates, Dye 2 exhibited stronger inhibitory effects on *Candida albicans* (MIC = 12.5 mg/ml) compared with Dye 1 (25 mg/ml). Both dyes, however, displayed equal inhibitory activity against *Aspergillus niger* (25 mg/ml each). The higher antifungal potency of Dye 2 may be attributed to its higher content of phenolic or carbonyl functional groups, which are known to interact with fungal cell wall sterols and disrupt membrane integrity [44,45]. Such phytochemical interactions can cause leakage of vital cellular constituents and inhibition of enzymatic systems essential for fungal growth.

Overall, the MIC findings demonstrate that both dyes possess broad-spectrum antimicrobial capabilities, though their performance varies with microbial type. The consistency between the MIC data and inhibition-zone results further reinforces the reliability of the observed antimicrobial trends. Dye 2 shows generally stronger activity against fungal species, while Dye 1 appears slightly more effective against *E. coli*. These complementary properties suggest potential synergistic applications of both dyes in developing eco-friendly heterocyclic dyes for antimicrobial coatings, textiles, and leather materials that combine aesthetic coloration with protective functionality [46,47].

Table 9 Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) in mg/ml

Test Organism	Dye 1	Dye 2
Staphylococcus aureus	12.5	12.5
Escherichia Coli	50	50

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Test Organism	Dye 1	Dye 2
Candida albicans	50	12.5
Aspergillus niger	50	25

Key: ND = No detection
— = No activity

The Minimum Bactericidal/Fungicidal Concentration (MBC/MFC)

The Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) values presented in Table 3 highlight the effectiveness of the two natural dyes against selected bacterial and fungal organisms. Both dyes exhibited notable antimicrobial properties, though their activity varied depending on the microbial species tested. This variability indicates that the dyes' efficacy is organism-dependent and influenced by microbial cell wall architecture and permeability differences [48,49].

Among the bacterial isolates, *Staphylococcus aureus* (Gram-positive) showed greater sensitivity to both dyes, with the lowest MBC value of 12.5 mg/ml, while *Escherichia coli* (Gram-negative) displayed reduced susceptibility, with an MBC of 50 mg/ml. This difference is consistent with previous findings that Gram-negative bacteria possess an additional outer membrane composed of lipopolysaccharides, which restricts the entry of large or hydrophobic compounds such as plant-derived dye molecules [50,51]. Conversely, the simpler peptidoglycan structure of Gram-positive bacteria allows easier penetration of active dye constituents, resulting in stronger bactericidal effects.

Similarly, the fungal isolates *Candida albicans* and *Aspergillus niger* responded differently to the dyes. *C. albicans* showed high sensitivity to Dye 2 (MFC = 12.5 mg/ml) compared to Dye 1 (50 mg/ml), while *A. niger* exhibited moderate inhibition, with MFC values of 50 mg/ml for Dye 1 and 25 mg/ml for Dye 2. The higher antifungal activity of Dye 2 could be attributed to its richer composition of functional groups, such as hydroxyl or carbonyl moieties, which may enhance its ability to complex with fungal cell wall proteins and interfere with ergosterol biosynthesis [52,53].

CONCLUSION

The present study highlights the successful synthesis and characterization of dyes D1 and D2, both obtained with satisfactory yields of 70% and 80% and relatively high melting points, confirming their purity and stability. FT–IR analysis confirmed the presence of expected functional groups, with both dyes exhibiting characteristic hydroxyl, amino, aromatic, and azo (–N=N–) bands, alongside sulfonate groups that enhance solubility and fiber affinity. UV–Visible spectroscopy revealed distinct optical properties, with D1 showing an absorption maximum at 473 nm, while D2 exhibited a bathochromic shift to 488 nm, indicating stronger conjugation and improved electronic interactions.

These optical characteristics contributed to improved performance properties, particularly in fastness and antimicrobial activity. Dye D2 demonstrated superior wash and light fastness ratings, as well as higher antimicrobial activity, including larger inhibition zones and lower MIC values against selected organisms. Overall, the enhanced conjugation and substituent effects in D2 significantly improved its colour strength, stability, and biological activity, establishing it as a more effective multifunctional dye for textile and related applications.

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