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SYNTHESIS OF SOME AZO COMPOUNDS THROUGH COUPLING REACTIONS AND THEIR BIOLOGICAL EVALUATION

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ABSTRACT

Five azo compounds where synthesized by coupling reaction between diazonium salt obtained from aniline and some aromatic compounds. The azo compounds obtained from 1-naphthol and 2-naphthol have excellent yields of 96% and 81% respectively while that obtained from phenol has a moderate yield of 58%. The azo compounds obtained from benzene and toluene on the other hand have shown poor yields of the products (4% and 11% respectively).Structures of the compounds were confirmed by FT-IR, ^{1}H NMR, ^{13}C NMR & UV-Visible. All the azo compounds were screened for bioactivity against *Streptococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli*. The results obtained showed that the azo dye synthesized from coupling aniline and 2-naphthol (coded as C-5) showed the highest activity against the three test organisms. At concentrations of 50µg/ml - 100µg/ ml it has an average inhibition zone of 22mm. *Escherichia coli* showed the highest resistance against all the tested azo compounds. The azo compounds obtained from coupling aniline with benzeneortoluene are completely inactive against *E. coli* while others have weak activity against it.

KEYWORDS

Diazonium salt, Azo compounds, Bioactivity, *Streptococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli*.

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INTRODUCTION

Azo compound is any organic chemical compound having azo (-N=N-) functional group or chromophore as part of its molecular structure. The groups attached to the nitrogen atoms may be of any organic class, but most azo compounds have aromatic rings attached to the azo group. These compounds have ability to absorb light and appear coloured hence they are largely used as commercial

dyes. For this reason azo compounds are mostly used in the industry as synthetic colourants and used as acid-base indicators in chemistry laboratories[1].

Apart from their cheap functions as dyes, azo compounds have been investigated for their pharmaceutical usage. For example, prontosil was found to protect against and cure streptococcal infection in mice[2]. The azo dye sulfonamides are also antibacterial drugs that are used systematically for the cure of bacterial infections in humans[3]. Presently, the sulfonamides-trimethoprin combination is used extensively for opportunistic infection in AIDS patients[2]. It was also reported that 4-phenylazophenoxyacetic acids have shown antimicrobial activity against two Gram-positive bacteria *Staphylococcus aureus* and *Streptococcus pyogenes* as well as three Gram-negative bacteria *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Escherichia coli*[4-6]. Azo Schiff bases were also shown to exhibit antibacterial activity against *Bacillus subtilis* and antifungal against several fungi[7].

In continuation of the search for the pharmaceutical advantages of the azo dyes our research group has synthesized five azo dyes by coupling reactions of anilinium diazonium salt with five aromatic compounds and screened them against one Gram-positive disease causing bacteria *Streptococcus faecalis* and two Gram-negative disease causing bacteria *Pseudomonas aeruginosa* and *Escherichia coli*. The results are hereby presented.

EXPERIMENTAL

All solvents were obtained dry from a Grubbs dry solvent system and glassware was flame dried and cooled under vacuum before use. ^{1}H and ^{13}C NMR spectra were measured using $CDCl_3$ as solvent on a Bruker 250MHz machine. Chemical shifts for carbon and hydrogen are given on the scale relative to TMS (tetramethylsilane, δ = 0ppm). ^{13}C NMR spectra were recorded using the JMOD method. IR spectra were recorded on a Perkin-Elmer 1600 FT-IR machine using 0.5mm NaCl cells.

λmax were found from measurements of absorptions at various wavelengths on Model 721 spectrophotometer. Melting point was carried out at room temperature using Gillen Kamp melting point apparatus. Bioactivity was carried out using standard agar (Mueller-Hinton agar and Nutrient Broth).

General procedure for the synthesis of the azo compounds[2]

Aniline (1 equivalent) was dissolved in 16 ml of concentrated HCl and 16ml of water was immediately added to the solution in beaker and cooled in crushed ice. A solution of $NaNO_3$ (1 equivalent) in 20 ml of water was added with stirring to generate the diazonium salt *in situ*. A solution of the aromatic compound (1 equivalent) in 45ml of 10% NaOH was prepared in 250 ml beaker and cooled to 5^{o}C by immersion in an ice bath with 25 g of crushed ice. The cold diazonium salt solution prepared above was slowly added to the cold solution of the aromatic compound with stirring until it was exhausted. The mixture was allowed to stand in an ice bath for 30 minutes with occasional stirring. The coloured precipitate formed was filtered by suction, dried and weighed.

Diphenyldiazene: $C_{12}H_{10}N_2$ (compound C-1)

Using the general procedure above, starting from 5.0 g (4.9 ml, 0.054mol) of aniline and 4.0g (0.054mol) of $NaNO_3$, the corresponding diazonium salt was generated *in situ* at ice bath temperature and coupled immediately with 4.20g (0.054mol) of benzene to obtain the title compound as coffee coloured crystals (0.8g, 4% yield), melting point 60-62^{o}C, FT-IR 1599cm^{-1} (-N=N-) and λ_{max}= 320nm. ^{1}H NMR (250 MHz; $CDCl_3$) δ_H7.39 (4H, d, Ar-H), 7.46 (6H, d, Ar-H); ^{13}C NMR (100 MHz; $CDCl_3$) δ_C122.7 (4 × Ar*C*H), 128.8(4 × Ar*C*H), 130.7(2 × Ar*C*H), 152.5 (2 × Ar*C*-N=N).

4-methyldiphenyldiazene: $C_{13}H_{12}N_2$ (compound C-2)

Using the general procedure above, starting from 5.0g (4.9ml, 0.054mol) of aniline and 4.0g (0.054mol) of $NaNO_3$, the corresponding diazonium salt was generated *in situ* at ice bath temperature and coupled immediately with 4.97g (0.054mol) of toluene to obtain the title compound as dark yellow crystals (1.8g, 11% yield), melting point 90-92^{o}C, FTIR 1603cm^{-1} (-N=N-) and λ_{max}= 380nm. ^{1}H NMR

(250 MHz; $CDCl_3$) δ_H2.35 (3H, s, C*H*$_3$), 7.26 (2H, d, Ar-*H*), 7.46 (3H, d, Ar-*H*), 7.81-7.93(4H, m, Ar-*H*); ^{13}C NMR (100 MHz; $CDCl_3$) δ_C21.0 (*C*H$_3$), 122.6 (2 × Ar*C*H), 123.7 (2 × Ar*C*H), 128.8 (2 × Ar*C*H), 129.6 (2 × Ar*C*H), 130.7 (Ar*C*H), 139.6 (Ar*C*), 149.5 (Ar*C*-N=N), 152.2 (Ar*C*-N=N).

4-(phenyldiazenyl) phenol: $C_{12}H_{10}N_2O$ (compound C-3)

Using the general procedure above, starting from 5.0g (4.9ml, 0.054mol) of aniline and 4.0 g (0.054mol) of $NaNO_3$, the corresponding diazonium salt was generated *in situ* at ice bath temperature and coupled immediately with 10.69g (0.054mol) of phenol to obtain the title compound as dark green crystals (5.4g, 58% yield), melting point 138-140^{o}C, FTIR 1588cm^{-1} (-N=N-) and λ_{max}= 380nm. ^{1}H NMR (250 MHz; $CDCl_3$) δ_H 5.37 (1H, s, O*H*), 6.93 (2H, d, Ar-*H*), 7.46 (3H, d, Ar-*H*), 7.76 (2H, d, Ar-*H*), 7.93 (2H, d, Ar-*H*); ^{13}C NMR (100 MHz; $CDCl_3$) δ_C 116.0 (2 × Ar*C*H), 122.7 (2 × Ar*C*H), 124.1 (2 × Ar*C*H), 128.8 (2 × Ar*C*H), 130.7 (Ar*C*H), 145.1 (Ar*C*-N=N), 152.5 (Ar*C*-N=N), 159.6 (Ar*C*).

4-(phenyldiazenyl)-1-naphthol: $C_{16}H_{12}N_2O$ (compound C-4)

Using the general procedure above, starting from 5.0g (4.9ml, 0.054mol) of aniline and 4.0g (0.054mol) of $NaNO_3$, the corresponding diazonium salt was generated *in situ* at ice bath temperature and coupled immediately with 13.39g (0.054mol) of 1-naphthol to obtain the title compound as maroon coloured crystals (12g, 96% yield), melting point 160-162^{o}C, FTIR 1596 cm^{-1} (-N=N-) and λ_{max}= 420nm. ^{1}H NMR (250 MHz; $CDCl_3$) δ_H 5.0 (1H, s, O*H*), 6.84 (1H, d, Ar-*H*), 7.38-7.84 (9H, m, Ar-*H*), 8.10 (1H, d, Ar-*H*); ^{13}C NMR (100 MHz; $CDCl_3$) δ_C 109.0 (Ar*C*H), 120.0 (Ar*C*H), 121.4 (Ar*C*H), 122.7 (2 × Ar*C*H), 124.6 (Ar*C*), 126.2 (2 × Ar*C*H), 127.6 (Ar*C*H), 128.8 (2 × Ar*C*H), 129.2 (Ar*C*), 130.7 (Ar*C*H), 144.7 (Ar*C*-N=N), 152.6 (Ar*C*-N=N), 153.7 (Ar*C*-O).

4-(phenyldiazenyl)-2-naphthol: $C_{16}H_{12}N_2O$ (compound C-5)

Using the general procedure above, starting from 5.0g (4.9ml, 0.054mol) of aniline and 4.0g (0.054mol) of $NaNO_3$, the corresponding diazonium salt was generated *in situ* at ice bath temperature and coupled immediately with 13.39g (0.054mol) of 1-naphthol to obtain the title compound as maroon coloured crystals (10.8g, 81% yield), melting point 133-135^{o}C, FTIR 1599cm^{-1} (-N=N-) and λ_{max}= 400nm. ^{1}H NMR (250 MHz; $CDCl_3$) δ_H 5.0 (1H, s, O*H*), 7.18 (1H, s, Ar-*H*), 7.21 (1H, t, Ar-*H*), 7.30-7.93 (9H, m, Ar-*H*); ^{13}C NMR (100 MHz; $CDCl_3$) δ_C 118.0 (Ar*C*H), 112.6 (Ar*C*H),122.7 (2 × Ar*C*H), 123.4 (2 × Ar*C*H),124.6 (Ar*C*), 126.3 (Ar*C*H), 127.7 (Ar*C*H), 128.8 (2 × Ar*C*H), 129.2 (Ar*C*), 130.7 (Ar*C*H), 144.7 (Ar*C*-N=N), 152.6 (Ar*C*-N=N), 153.6 (Ar*C*-O).

Antimicrobial Test

The antimicrobial test was carried out by an adapted agar (Mueller-Hinton agar &Nutrient Broth). The antimicrobial activity of the synthesized compounds was tested against *Streptococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli* microbial strains according to the following procedure[8]:

Preparation of the Medium

The nutrient agar medium was prepared by dissolving 9.0g of agar in 500ml of distilled water in a conical flask and swirled to dissolve. The solution was sterilized in an autoclave at 121^{o}C for 15min. It was then poured aseptically into petri dishes, allowed to solidify and set for the analysis.

Preparation of the Hydrazone samples

The concentrations of the hydrazone compounds were prepared by serial dilution. 0.5g of each compound was dissolved in 0.5ml of dimethyl sulfoxide (DMSO) to yield a concentration of 1.0g/ml equivalent to 10^6µg/ml as stock solution. From the stock solution, 0.1ml was transferred into a sterile bijou bottle containing 0.9ml of DMSO thus giving a concentration of 10^5µg/ml. From this solution 0.1ml was transferred into another sterile bijou bottle containing 0.9ml of DMSO which gave a concentration of 10^4µg/ ml and this was further diluted to 1000µg/ml, 100µg/ml, 50µg/ml and 12.5µg/ml.

Preparation of culture medium and inoculation

Cultures of *Streptococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli* were obtained from Bayero University, Department of microbiology. Pure isolates were obtained by sub-

culturing unto fresh nutrient agar plates. The freshly grown microbial cultures were appropriately diluted in test tubes containing sterile normal saline solution to march McFarland standard described by Cheese brough M. (2000)[8]. The McFarland standard was prepared by mixing 0.6ml of 1% (W/V) dehydrated barium chloride solution with 99.4ml of 1% (V/V) sulphuric acid solution and was labeled as the standard inoculums. The standard inoculums were then evenly smeared onto the prepared nutrient agar plates. After smearing, plates were dried for 15min, and wells were punched using sterile cork borers. Once wells were formed, they were filled with concentrations of plant extracts. Commercially available ciprofloxacin (500mg) was used as positive control in this study. Plates were inoculated for 24hours at 37^{o}C to allow extracts to diffuse through the agar media to form a zone of inhibition. The diameters of the zone of inhibition for different extracts against the different bacteria were measured in millimeter for further analysis. An agar well (6mm) showing no zone of inhibition was considered as no antimicrobial activity.

Determination of Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration of the hydrazone samples were prepared by serial dilution using distilled water to obtain concentrations of 10µg/ml, 8µg/ml, 6µg/ml and 4µg/ml.

Equal volume (2ml) of the hydrazone sample and Nutrient broth were mixed. Specifically 0.1ml of standardized inoculation (3.3x106 CFU/ml) was added to each of the test tubes above. The tubes were inoculated aerobically at 37^{o}C for 24hours. Tubes containing broth and thehydrazone samples without inoculation served as positive control while tubes containing broth and inoculation served as negative control. The tubes were observed after 24hours of incubation to determine minimum inhibitory concentration; that is the lowest concentration that showed no evidence of growth[9].

RESULTS AND DISCUSSION

The general method for the synthesis of the azo compounds is represented in Scheme No.1. The first step of the reaction is the formation of the aromatic diazonium salt at ice-bath temperature because of the instability of the compound. This was achieved by reacting the aniline with $NaNO_2$ in conc. HCl at 0^{o}C. The sodium nitrite first reacts with hydrochloric acid to generate nitrous acid (HNO_2) according to the equation below:

$$HCl + NaNO_2 \longrightarrow HNO_2 + NaCl$$

The nitrous acid then converts the aniline into the diazonium salt. The diazonium salt generated *in situ* was immediately coupled with the aromatic compound at 0^{o}C temperature. All the five compounds were obtained as coloured crystals with compounds C-4 and C-5 derived from the two positional isomers of naphthol having excellent yields (Table No.1). Compound C-3 derived from phenol has moderate yield of 58%. The other two azo compounds derived from benzene and toluene have poor yield of the products. It was well known that coupling reactions with diazo compounds are aided by strong electron donating substituents at the *para* position of the aromatic compound which might be the reason of the higher yield in the phenol and naphthol derivatives.

The structures of the azo compounds were established by spectroscopic analysis. Their FT-IR spectra showed signals around 1588-1603 cm^{-1} characteristic of N=N stretching vibrations. All the ^{1}H and ^{13}C NMR spectra agreed with the structures as indicated under Experimental section.

The antimicrobial activity of the azo compounds showed different trend of activity against *Streptococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli* (Table No.2). Ciprofloxacin was used as a reference drug. Generally the activity increases with the increasing concentrations. All the five azo compounds showed activity against *Streptococcus faecalis* with compound C-5 showing the highest activity at 100µg/ml having a zone of inhibition of 27mm. It is significant to note that even when the concentration drops to 50µg/ml, the activity of compound C-5 against *Streptococcus faecalis* remains the same and only slightly drops at much lower concentrations of 25 and 2.5µg/ml. The rest of the compounds showed good activity against *Streptococcus faecalis* but the activity suddenly

drops at lower concentrations. In comparison the azo compounds C-1, C-3 and C-4 have a similar trend of activity against *Pseudomonas aeruginosa.* They have an average zone of inhibition of 20mm at 100µg/ml that suddenly drops at lower concentrations. Compound C-5 showed good activity against *Pseudomonas* which only slightly drops at lower concentrations. Compound C-2 is completely inactive against *Pseudomonas aeruginosa* at all concentrations. *Escherichia coli* showed the highest resistance against all the tested azo compounds. Compounds C-1 and C-2 are inactive to the strand at all tested concentrations. Compound C-3 has only 10mm of zone of inhibition at 100µg/ml which drops to in activity at lower concentrations while C-4 and C-5 have 20mm zone of inhibition at 100µg/ml but becomes inactive at 25µg/ml and lower concentrations. Generally, compound C-5, the azo dye synthesized by coupling reaction between aniline and 2-naphthol showed the highest activity against the three test organisms. Most of the compounds have MIC of 4µg/ml against the tested organisms (Table No.3).

Table No.1: Physical properties of the azo compounds

S.No	Compounds	Colour	% Yield	Melting Point (ºC)
1	C-1 (R = benzene)	Coffee colour	4%	60-62
2	C-2 (R = toluene)	Dark yellow	11%	90-92
3	C-3 (R = phenol)	Orange	58%	138-140
4	C-4 (R = 1-naphthol)	Maroon	81%	158-160
5	C-5 (R = 2-naphthol)	Reddish brown	96%	133-134

Table No.2: Result of the antimicrobial activity of the synthesized AZO compounds

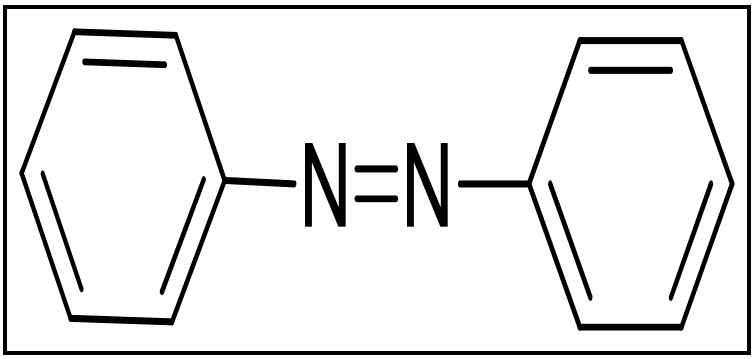
S.No	Product	Organism	Concentration / Diameter of Zone of Inhibition (mm)				
			100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	Control
1	C-1	*Streptococcus faecalis*	15	14	13	12	34
		Pseudomonas aeruginosa	20	15	11	10	34
2		*Escherichia coli*	00	00	00	00	34
3	C-2	*Streptococcus faecalis*	18	17	14	12	35
		Pseudomonas aeruginosa	00	00	00	00	35
4		*Escherichia coli*	00	00	00	00	35
5	C-3	*Streptococcus faecalis*	25	23	22	17	30
		Pseudomonas aeruginosa	20	10	10	09	30
6		*Escherichia coli*	10	05	00	00	30
7	C-4	*Streptococcus faecalis*	20	22	19	20	30
8		*Pseudomonas aeruginosa*	17	20	15	15	30
9		*Escherichia coli*	20	17	00	00	30
10	C-5	*Streptococcus faecalis*	27	27	25	20	33
11		*Pseudomonas aeruginosa*	22	21	17	17	33
12		*Escherichia coli*	20	20	00	00	33

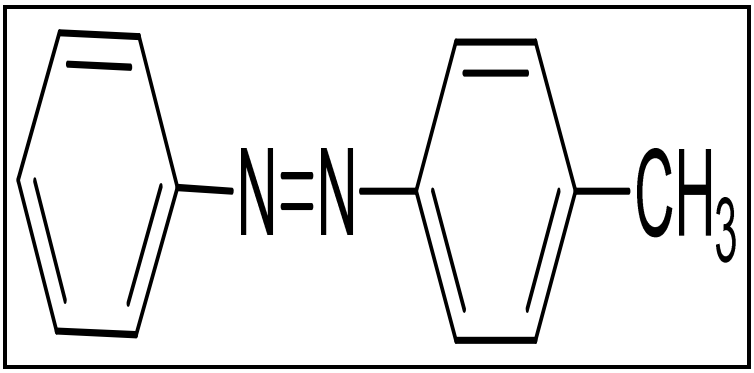
Inactive (inhibition zone <6mm)

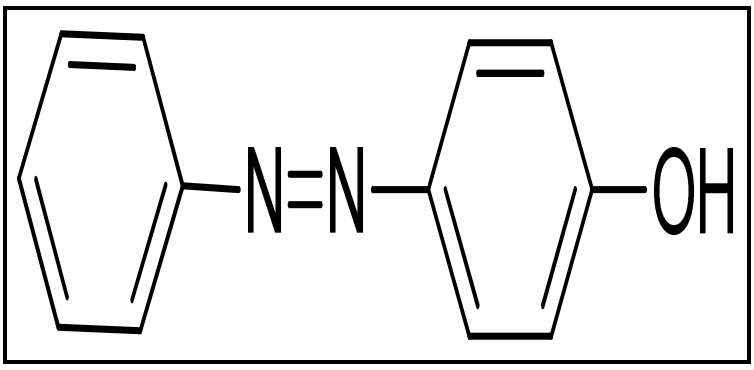
Table No.3: Minimum Inhibition Count (MIC) of the synthesized azodyes against *Streptococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli*

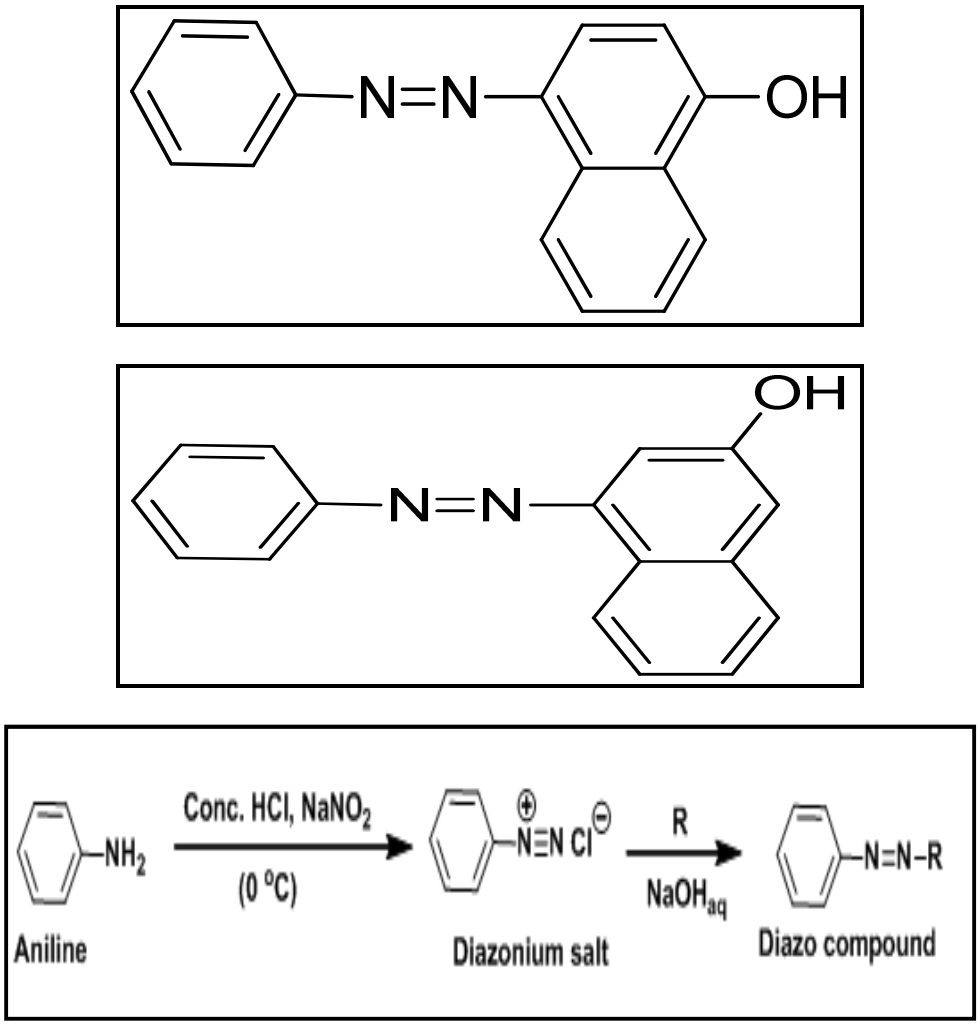
S.No	Compounds	*Streptococcus faecalis*				*Pseudomonas aeruginosa*				*Escherichia coli*			
		10 µg/ml	8 µg/ml	6 µg/ml	4 µg/ml	10 µg/ml	8 µg/ml	6 µg/ml	4 µg/ml	10 µg/ml	8 µg/ml	6 µg/ml	4 µg/ml
1	C-1	-	-	-	+	-	-	-	+	-	-	-	-
2	C-2	-	-	-	+	-	-	-	-	-	-	-	-
3	C-3	-	-	-	+	-	-	+	-	-	-	-	-
4	C-4	-	-	-	+	-	-	-	+	-	-	-	-
5	C-5	-	-	-	+	-	-	-	+	-	-	-	+

+ = Growth; - = No growth









Scheme No.1: Synthesis of the diazo compounds

CONCLUSION

In conclusion, five azo dyes have been synthesized by diazotization of aniline and subsequent coupling reactions of the resultant aromatic diazonium salt with aromatic compounds at lower temperatures. Two azo dyes obtained from 1-naphthol and 2-naphthol were obtained at excellent yields of 81 and 96% respectively. The azo compound obtained from phenol has a moderate yield of 58% while those obtained from benzene and toluene have poor yields of 4% and 11% respectively. The assigned structures of the compounds were supported by FT-IR, ^{1}H NMR and ^{13}C NMR. The compounds were screened for activity against three disease causing pathogens *Streptococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli* using Mueller-Hinton agar and Nutrient Broth method. Compound C-5, the azo dye synthesized by coupling reaction between aniline and 2-naphthol showed the highest activity against the three test organisms at the tested concentrations; however other azo compounds showed some promising results as well. *Escherichia coli* showed resistance to the three azo dyes synthesized from benzene, toluene and phenol.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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