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Full Length Research Paper

Antimicrobial properties of 4-Carboxyl-2, 6-dinitrophenylazohydroxynaphthalenes

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The antimicrobial screening of the monoazo dyes, 4-carboxyl-2, 6-dintropheylazohydroxynapththalene, was carried out as a preliminary test for evaluating their biological potentials. Antimicrobial screening was done with filter paper disc and gauze bandage fabric impregnated with the dyes using 7 microbial isolates: *Staphylococcus aureus* 382, *Candida albicans* UBA7, *Klebsiella* species 864, *Escherichia coli* V_3, *Salmonella enterica gallinarum* 28 V_2, *Pseudomonas aeruginosa* and *Proteus* species. Diameters of zones of inhibitions were measured to evaluate the activities of the dyes. The minimum inhibitory concentration of the four title dyes was thereafter estimated using two sensitive strains of *S. aureus* and *Candida albicans*. AZ-01 and AZ-04 at a concentration of 5 mg/mL was found to have the highest activities of 20 mm each on *S. aureus*, comparable to the positive control gentamicin (diameter of 20 mm), using paper disc. With the fabric screening using 5 mg/mL and 10 mg/mL concentration, only the 5 mg/mL AZ-01 and the 10 mg/mL AZ-01 had activities of 10 and 12 mm respectively on *S. aureus*. On *C. albicans*, 5 mg/mL AZ-01 and 10 mg/mL AZ-01 had activities of 20 and 24 mm respectively and these were found comparable to the positive control 1% tioconazole. 5 mg/mL AZ-02 and 10 mg/mL AZ-02 had activities of 10 and 14 mm respectively, 5 mg/mL AZ-04 and 10 mg/mL AZ-04 had activities of 16 and 16 mm, respectively. The minimum inhibitory concentration (MIC) done using serial dilution on WHO microtitre plate (96 wells), gave AZ-01 having the highest activity, both on *S. aureus* and *C. albicans* with MIC of 15.63 µg/mL, and then AZ-04 with MIC of 23.44 µg/mL. The results of these microbial assays suggest that the investigated monoazo dyes may be useful as potential antimicrobial agents, especially against multidrug resistant *S. aureus*, in addition to their dyeing properties.

Key words: 4-carboxyl- 2, 6-dinitrophenylazohydroxynaphthalenes, antimicrobial properties, multidrug resistant *Staphylococcus aureus*, MIC.

INTRODUCTION

Antimicrobial properties of incorporated dyes in food, beverages, cosmetics and fabrics become desirable as that prolong the utility of such products by inhibiting bacterial and fungal growth. Dixit B.C. Patel and Desai, 2007 worked on the antimicrobial and fabric-protective activities of naphthol- based azo dyes using 2,4-dihydroxybenzophenone, an aromatic ketone (diphenyl ketone), as an important starting material in organic synthesis of dyes. They showed that the antimicrobial properties of these azo dyes reduced and prevent damages made to the mechanical strength of materials, colour changes, stains and stale odour. Azo dyes produced from naphthols and phenol has wide applications as polymer additives (Yurteri et al., 2002). Their hydroxyl groups are good UV light absorbers which

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prevent photo degradation of most vinyl polymers (Allmer et al., 1989). Baskar et al., 2006 found out that various naphthol dyes e.g. naphthoxazole and nerolins possess outstanding biological activities like anti-amnesic, anti-inflammatory, analgesic as well as antibacterial and antifungal properties, by using Agar diffusion method against *S. aureus, B. subtilis P. aeruginosa, E. coli* and *A. niger*. Zone of inhibition recorded compared with standard antimicrobial agents such as ciprofloxacin, chloramphenicol and clotrimazole. Naphathoxazole showed the best activity against all strains including fungi, while others showed comparative and moderate activities against all bacteria strains. Abedi et al. (2008) found out that acrylic fabric treated with direct dye (CI direct blue 168) using $CuSO_4$ as mordanting agent showed considerable antimicrobial properties against common pathogenic bacteria. Activities increased with increase in temperature and using 2% $CuSO_4$ in pre- and post-mordanting scenarios. Also, bactericidal triazine dyestuffs impart the ability to inhibit growth of bacterial, *S. aureus* while azo dyestuffs was developed to impart fungicidal properties to protein fibrous materials, especially under tropical climate, that is, at increased humidity and elevated temperature (Gorbacheva, 1978).

Untreated azo dyes from industrial water waste was found to have a high toxic effect on soil bacterium, *P. aeruginosa*, while treated dyes (Decolourised) has reduced toxicity because of decrease in concentration, while high concentration of azo dyes eliminated microbial colonies due to high frequency of mutation. This last 2 reports show that the higher the concentration of azo dyes, the higher would be the toxic or antimicrobial effect. Indole and its azo dye derivatives under aerobic condition exhibit toxic activity on both pathogenic and non-pathogenic micro organisms; *B. thuringiensis, B. subtilis, B. megaterium, P. aeruginosa, S. aureus, E. coli and S. cerevisiae* (O'ztu¨rk et al., 2006).

Some azo dyes have been found to have antimicrobial properties due to the presence of active functional groups. This biological activity of some azo dyes has increased their usage in textile, foods, and pharmaceutical industries, since they have this vital ability to inhibit microorganisms which cause degradation to textile materials, degradation to food materials, skin diseases produced from bacteria which live inside clothing and textile fabrics and other ailments that affect human health. A search for azo dyes with potent antimicrobial properties is still on-going. 4-carboxy-2, 6-dinitrophenylazohyroxynaphthalenes series are therefore being investigated as potential antimicrobial agents due to the presence of phenolic functional group in these dyes. These dyes were synthesized from various naphthol derivatives (Adegoke et al., 2008). Further attempts at some physicochemical characterizations were carried out using various hydrophobicity parameters and subtle differences were discovered in their migration patterns on thin layer chromatography (Idowu et al., 2007). The solvatochromic behaviours and structure-spectra properties of the dyes have also been recently reported (Adegoke and Idowu, 2010).

This paper reports the first biological evaluation of these highly functionalised azo dyes as preliminary tests towards other bioactivity assessment and structural modifications.

MATERIALS AND METHODS

Chemicals and reagents

-naphthol, -naphthol (Merck), chloroform, Analytical grade ethanol, methanol and acetone, glacial acetic acid, *n*-hexane, ethyl acetate, dimethylformamide (BDH chemical limited, Poole, England), naproxen and nabumetone crystals (Sigma-Aldrich chemicals USA).

Materials for antimicrobial screening

Filter paper discs (Whatman), sample vials, gauze bandage (fabric), perforator (Bliss single hole punch), desiccators, spatula, petri dishes, Oxoid Muller-Hilton agar batch cmo337 (Oxoid), Nutrient broth (Oxoid), Pipetman microliter pipette (Gilson, France), Tryptone soy broth TSB (Oxoid), pipette tips, 0-200 µL, (Fisher brand scientific), WHO microtitre plates (Linbro).

Microorganisms isolates used

S. aureus (382): A clinical isolate from University College Hospital (UCH), Ibadan, Nigeria; UBA7 *C. albicans*: A laboratory stock from the Department of Pharmaceutical Microbiology, University of Ibadan; 864 *Klebsiella* species: A clinical isolate from UCH; *E. coli* v3: A clinical isolate from Department of Veterinary Microbiology and Parasitology, University of Ibadan; 28 V2 *Salmonella Enterica gallinarum*: A clinical isolate from Department of Veterinary Microbiology and Parasitology, University of Ibadan; *P. aeruginosa*: A clinical isolate from UCH and *Proteus* species: A clinical isolate from UCH.

Azo dyes used

The following azo dyes were synthesized and used in this study: AZ-01 [6-hydroxy-5-(4-carboxy-2,6-dinitrophenylazo)-naphthalene]; AZ-02 [8-hydroxy-5-(4-carboxy-2,6-dinitrophenylazo)-naphthalene]; AZ-03 [6-hydroxy-5-(4-carboxy-2,6-dinitrophenylazo)-naphthalene-2-(propan-2-oic acid)] and AZ-04 [6-hydroxy-5-(4-carboxy-2,6-dinitrophenylazo)-naphthalene-2-(butan-2-one)].

Synthesis of the 4-carboxyl-2,6-dinitrophenylazohydroxynaphthelenes

The synthesis and batch-to-batch characterization of the four azo dyes utilized in this work were carried out as previously reported by Adegoke et al. (2008).

Antimicrobial screening test for the azo dyes

Preparation of impregnated filter paper discs

Filter paper disc was prepared using a perforator. About 100 pieces

of circular filter paper disc was measured to be 0.5 cm (5 mm). 10 mg of each dye was weighed inside a sample vial. 2 mL of DMF was added to each dye to make the concentration 5 mg/mL each. About 20 pieces of filter paper discs was put inside each vial, left to soak for about 45 min and then drained. The paper discs was then left to dry inside a desicator.

Antimicrobial screening test with paper disc

The *in vitro* antibiotic sensitivity test of *S. aureus* 382*, C. albicans* UBA7*, E. coli, P. aeruginosa, K. species* and *S. enterica gallinarum*, were carried out as described by Walton (1972) as modified by Adetosoye (1984) The isolated discrete colonies of each of the organisms was inoculated into 5 mL sterile nutrient broth and incubated at 37° C for 8 h. A 1:2000 dilution of the culture was made with sterile nutrient broth (0.01 mL of the culture was added to 4 mL sterile nutrient broth), and the mixture was shaken vigorously. Mueller-Hinton agar plates were inoculated by flooding with the 1:2000 diluted mixture of each of the bacterial and fungal culture. The excess fluid was drained off and the plate was allowed to stand on the bench for 1 h. Subsequently, the azo dye-treated filter papers were aseptically applied on the plates. It was allowed to stand on the bench for 1 h to allow the azo dyes to diffuse into the microbial inoculated agar. The plates were incubated at 37°C for 18 h after which the results were recorded. The zones of inhibitions around each azo dye were compared with that of the control antimicrobial agent (gentamicin).

Preparation of Gauze bandage fabric

The fabric was prepared using sterile scissors. About 200 pieces of square sized gauze bandage was prepared. The length and width was made to be 5 mm. Both 5 and 10 mg of each dyes was weighed into sample vials. 1 mL of dimethylformamide was added to each vials, and the mixture shaken very well to dissolve. About 20 pieces of the prepared gauze bandage fabrics was then added to the solution. This was left for about 30 minutes and then drained. The fabric was left to dry inside a desicator.

Antimicrobial screening test using impregnated fabrics

The *in vitro* antibiotic sensitivity test of *S. aureus* 382*, C. albicans* UBA7*, E. coli, P. aeruginosa, Klebsiella species* and *S. enterica gallinarum*, were carried out as described by Walton (1972) as modified by Adetosoye (1984). The isolated discrete colonies of each of the organisms was inoculated into 5 mL sterile nutrient broth and incubated at 37°C for 8 h. A 1:2000 dilution of the culture was made with sterile nutrient broth (0.01 mL of the culture was added to 4 mL sterile nutrient broth), and the mixture was shaken vigorously. Mueller-Hinton agar plates were inoculated by flooding with the 1:2000 diluted mixture of each of the bacteria and fungus culture. The excess fluid was drained off and the plate was allowed to stand on the bench for 1 h. Subsequently, the azo dye impregnated fabrics were aseptically applied on the plates. It was allowed to stand on the bench for 1 h to allow the azo dyes to diffuse into the microbial inoculated agar. The plates were incubated at 37°C for 18 h after which the results were recorded. The zones of inhibition around each azo dye were compared with that of the control antimicrobial agent.

Determination of minimum inhibition concentration (MIC)

Preparation of dyes and isolates

10 mg of each of the four azo dyes was weighed into four sample vials. 1 mL of DMF was added and shaken to dissolve to make 10 mg/mL. A colony of each isolates was inoculated into 5 mL sterile nutrient broth and incubated at 37°C overnight.

Procedure used to determine the minimum inhibitory concentration MIC of the azo dyes with activities.

The minimum inhibitory concentration MIC of azo dyes 01, 02, 03 and 04 for isolates, *S. aureus* 382*,* and *C. albicans* UBA7, that produced antimicrobial activities from the antimicrobial screening test were determined by microtitre methods as described by Adetosoye and Rotilu (1987). 5 and 10 mg of azo dyes 01, 02, 03 and 04 were respectively dissolved in 1 mL Dimethyl formamide DMF, to a final concentration of 5 and 10 mg/mL, respectively. Serial dilution was thereafter performed. 50 L of the dye under test was delivered to the first well. The mixture was thoroughly mixed and 50 L was transferred to the next well. The same procedure was carried out up to the twelfth well where 50 L of the mixture was discarded. Then 25 L of the overnight broth culture of the respective microorganism under investigation was respectively delivered to each well and 25 L of the indicator was added to each well.

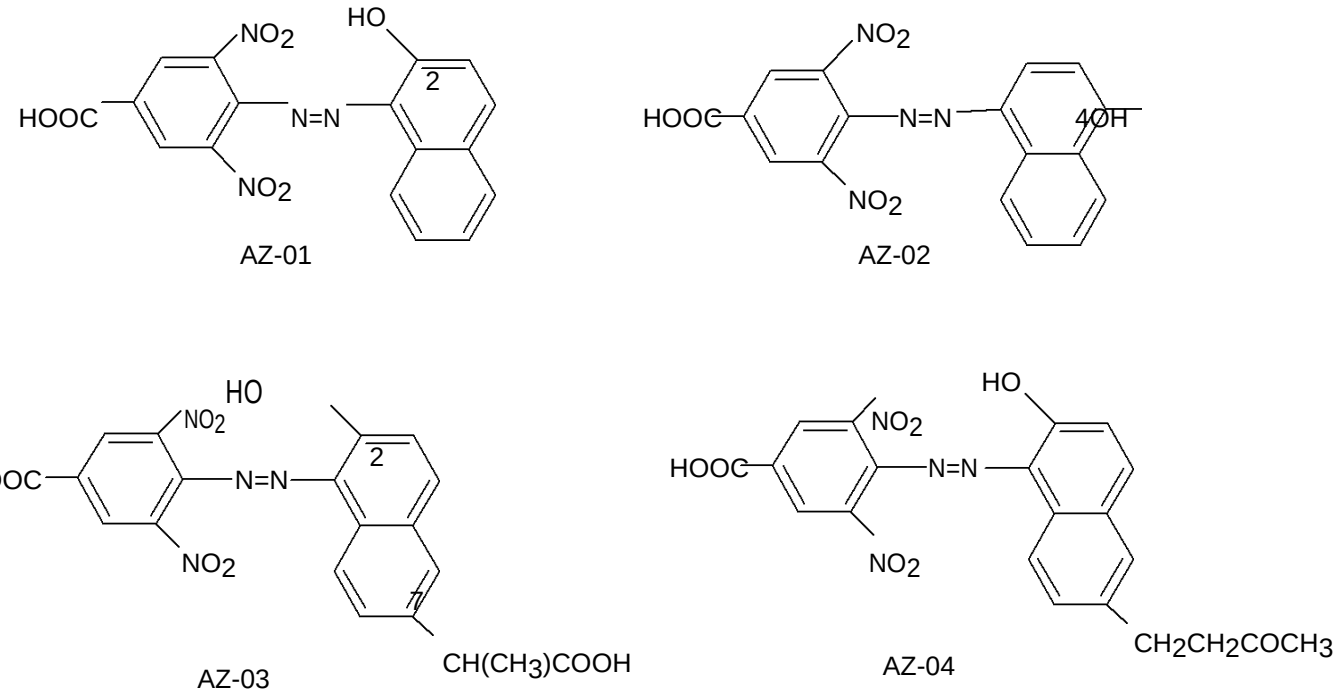
A negative control was set up containing 25 L of sterile TSB and 25 µL DMF. The microtitre plate was subsequently covered and incubated at 37°C for 18 h. The procedure was repeated for each of the azo dyes for the respective microorganisms. The MIC of the azo dye was taken as the lowest concentration of the dye that inhibits the growth of the microorganism. The well nearest to where there was no growth was taken as the Minimum inhibitory concentration of the azo dye under test.

RESULTS AND DISCUSSION

The structures of the 4-carboxyl- 2,6-dinitrophenylazohydroxynaphthalenes investigated in this work is presented in Figure 1. The azo dyes were screened for their antimicrobial activity against selected multi- drug resistant microbes [*S. aureus* 382, *C. albican*s (UBA 7), *Klebsiella* species 864, *E. coli* V3, *Salmonella* species 28 V2, *P. aeruginosa, P. mirabilis* and *S. Enterica gallinarum* 28 V2] using 5 mg/mL on paper disc.

The organisms selected such as *S. aureus*, is a pathogenic Gram-positive bacterium, which is the most frequently evaluated species, as it is the major cause of cross- infection in hospitals as well as in commercial and home laundry practices. It causes skin and tissue infections, septicaemia, endocarditis and meningitis and *E. coli* a Gram-negative bacterium, which is a popular test organism and is resistant to common antimicrobial agents. It causes urinary tract and wound infections, common in gastrointestinal tract and accounts for 25% of hospital infections (Bhat et al., 2005). The other organisms selected are also regular pathogenic organisms and are also implicated as indicator organisms for microbial contamination of food and pharmaceuticals.

The preliminary screening showed that all the azo dyes were effective against *S. aureus* 382 with azo dyes 01 and 04 having the highest activities with clear zone of inhibitions. Diameters of zones of inhibitions recorded are



Names of compounds

AZ-01: 4[(2-hydroxynapthalen-1-yl)diazenyl]-3,5-dinitrobenzoic acid

AZ-02: 4[(4-hydroxynaphthalen-1-yl)diazenyl]-3,5-dinitrobenzoic acid

AZ-03: 4[(7(1-carboyethyl)-2-hydroxynaphthalen-1-yl)diazenyl]-3,5-dinitrobenzoic acid

AZ-04: 4[(2-hydroxy-7-(3-oxobutyl)naphthalen-1-yl)diazenyl]-3,5-dinitrobenzoic acid

Figure 1. Structures of the dyes studied.

Table 1. Diameters of zones of inhibitions for the dyes on filter paper discs.

Organism	Zones of inhibitions (mm)				
	AZ-01	AZ-02	AZ-03	AZ-04	Negative control
S. aureus (382)	20	11	12	20	-
E. coli V3	-	-	-	-	-
P. aeruginosa	-	-	-	-	-
Klebsiella species 864	-	-	-	-	-
P. mirabilis	-	-	-	-	-
*S. Enterica gallinarum (*28 V2)	-	-	-	-	-
C. albicans	-	-	-	-	-

presented in Table 1. The azo dyes did not show any activity against the other microbes.

The four azo dyes were further screened for their antimicrobial activity using 5 mg/mL and 10 mg/mL on fabrics, since AZ-01 and AZ- 04 (5 mg/mL) showed good activity when screened with paper disc. The screening showed that AZ-01 alone was effective against *S. aureus* with increase activity as concentration increased to

Table 2. Minimum inhibitory concentrations of the title dyes on *Staphylococcus aureus* and *Candida albicans.*

Organism	MIC of dyes (µg/mL)			
	AZ-01	**AZ-02**	**AZ-03**	**AZ-04**
Staphylococcus aureus (382)	15.63	125	62.50	23.44
Candida albicans (UBA 7)	13.02	13.02	15.63	15.63

10 mg/mL. AZ-01, AZ-02 and AZ-04 were effective against *C. albicans* also with increased activity as concentration increased to10 mg/mL. There was no activity against the other microbes. The zones of inhibition recorded are presented in Table 2. AZ-04 has no activity, as compared to the 20 mm activity exhibited by both dyes when screened with paper disc.

Evaluation of the minimum inhibitory concentration of all the dyes was done in triplicate using three microtitre plate of 96 wells. The first clear well with plain colour as compared with the negative control wells indicated the well with the minimum inhibitory concentration. For *S. aureus*, AZ-01 is the most highly effective because it has the smallest MIC, 15.63 µg/mL than other dyes followed by AZ-04, 23.44 µg/mL. So the inhibitory effect of AZ-01 is the highest. Also with *Candida albicans* (UBA7), AZ-01 and AZ-02 has the minimum MIC's of 13.02 µg/ml, even lower than the amount required for the same amount of *S. aureus*. AZ-01 thus has been proven to be the most effective dye followed by AZ-04 from the estimation of the MIC (Table 3).

Generally, all the dyes were ineffective against the Gram-negative bacteria utilized while all showed varying degree of activity against the Gram-positive cocci, *S. aureus*. These observations may be due to structural differences between the bacteria. For Gram-positive bacteria, the main component of the cell walls is a rigid network composed of three macromolecular concentric shells, while Gram-negative bacteria have a network that is only one molecule thick, together with up to 25% (mass) of lipoprotein and lipopolysaccharide (Ma et al., 2003). Therefore, Gram-positive bacteria show greater resistance to mechanical rupture than Gram -negative cells. For example, Gram -positive bacteria can resist as high as 30 atmospheres of internal osmotic pressure; while for Gram- negative bacteria, the highest recorded internal osmotic pressure is only 8 atmospheres (Ma et al., 2003). The monoazo dyes investigated in this work due to their lipophilicity may have penetrated the cell of *S. aureus* readily than they will the cell wall of Gram-negative organisms. Previously reported agents against Gram-negative bacteria are usually those that can cause significant cell disruption by a surface active potential such as the cationic dyes reported by Ma et al. (2003) which damage the membrane through mechanical disruption and thereby exhibited stronger antimicrobial activities against Gram-negative cells (*E. coli*).

Structure-activity relationships

The dyes investigated in this work are closely related congeners with AZ-01 and AZ-02 being positional isomers. The common hydroxyl group in AZ-01 is located *ortho* to the azo linkage while in AZ-02 it is *para* to the azo linkage. AZ-03 and AZ -04 in addition to the common *ortho* hydroxyl substituent contains propionic acid and butanone moieties at the 7^{th} position. Our previous investigations (Adegoke et al., 2008; Idowu et al., 2007; Adegoke and Idowu, 2010) have demonstrated that AZ-01, -03 and -04 exist principally in the hydrazone tautomer. The azo-hydrazone tautomerism of these three congeners is presented in Figure 2. This structural imposition appears to have some influence on the

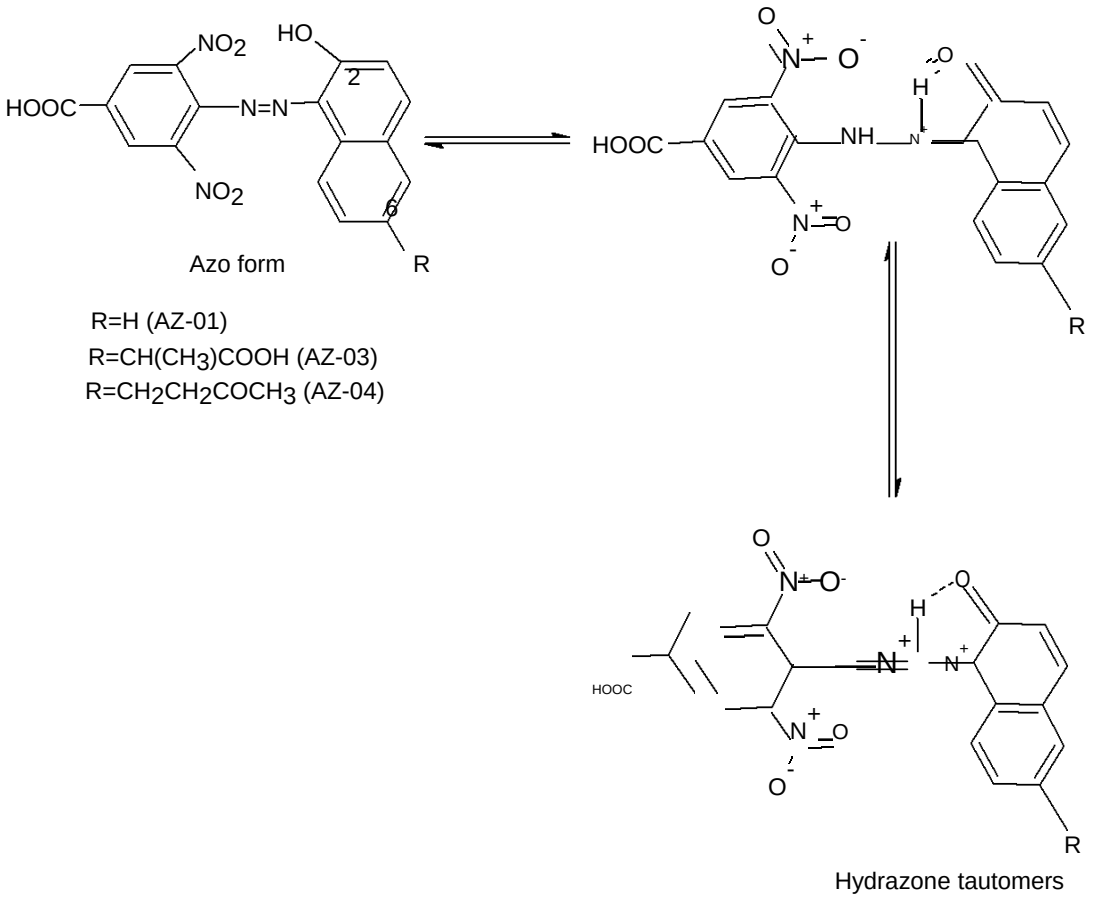


Figure 2. Azo-hydrazone tautomerism of AZ-01, -02 and -04.

observed antimicrobial properties investigated. While AZ-01 exhibited activities against both *Staphylococcus* and *Candida* both on disc and fabrics, the activities of AZ-03 and AZ-04 decreased on fabric. Thus, presence of the additional functional groups at the 7th position drastically affected the activity of the dyes. AZ-02 on the other hand did not exhibit significant antimicrobial activity. This is unexpected as it is anticipated that the presence of free hydroxyl group will aid antimicrobial potentials. However, the ability of the free hydroxyl group in AZ-02 to ionize might actually be preventing it from achieving enough intracellular concentration that will aid microbial kill. The abilities of the other three congeners to produce pseudo-rings (due to azo-hydrazone tautomerism) will obviously enhance their lipophilicity and hence increase transfer across cell membrane. The influence of this structural imposition is also reflected in the estimation of the MIC. AZ-01 produced the lowest concentration on *Staphylococcus* while the least effective once again in AZ-02. However, AZ-04 had better activity compared to AZ-03 suggesting that the butanone residue produced better lipophilic influence compared to the propionic acid residue. The sensitivity of *Candida* to the dyes (as observed for the fabric) was further reflected in the MIC estimations. All the dyes produced significantly low MIC values suggesting that the title dyes might be effective anti-Candida agents. The structural differences between AZ-01 and AZ-02 did not reflect on the MIC as both dyes gave the same MIC values against *C. albicans*. Further studies will be focussed on structural modifications of the monoazo dyes and in particular introduction of sulphonic acid moieties that will enhance water solubility.

Conclusions

The 4-carboxyl-2,6-dinitrophenylazohydroxylnaphthalene dyes exhibited wide-ranging activities especially against multi-drug resistant strains of *S. aureus* and the activities point to their potentials of being antimicrobial agents in addition to their dyeing properties.

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