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SYNTHESIS, CHARACTERISATION AND ANTIMICROBIAL SCREENING OF SOME AZO COMPOUNDS

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ABSTRACT: In this study, a series of azo compounds were synthesized in excellent yields via the diazotization of different aromatic amines followed by coupling with 2-naphthol. These compounds were characterized by elemental analysis, IR, ^{1}H NMR and ^{13}C NMR spectral techniques. The synthesized compounds have been tested in vitro against a number of microorganisms in order to assess their antimicrobial properties using disk diffusion method. The minimum inhibitory concentrations (MIC) were also determined by the broath microdilution technique. Some of the products exhibited comparable activity with known standard drugs at same concentration.

Keyword: 4-hydroxy-1-naphthalenesulphonic acid, Antimicrobial, Disc diffusion, Broath dilution.

INTRODUCTION

Nowadays, synthetic azo compounds are widely used in different application fields, such as medicines, cosmetics, food, paints, plastics, shipbuilding, automobile industry, cable manufacture, and in analytical chemistry[1-15]. Biological importance of azo compounds is well known for their use as antineoplastics, antidiabetics, antiseptics, anti-inflammatory, and other useful chemotherapeutic agents [16-19]. Azo compounds are known to be involved in a number of biological reactions such as inhibition of DNA, RNA and protein synthesis, carcinogenesis and nitrogen fixation [20, 21]. Evans blue and Congo Red are being studied as HIV inhibitors of viral replications. This effect is believed to be caused by binding of azo dyes to both protease and reverse transcriptase of this virus [22]. The existence of an azo moiety in different types of compounds has caused them to show antibacterial and pesticidal activity [23, 24]. Since compounds with azo moiety and naphthalene moiety have been extensively used as dyes ,but biological activity is less reported.

In the present work, we have synthesized and characterized six azo compounds namely phenylazo-2-naphthol (PAN), 1-[2-hydroxyphenylazo]-2-naphthol (HPAN), 1-[2-methoxyphenylazo]-2-naphthol (MPAN), 1-[2-chlorophenylazo]-2-naphthol (ClPAN), 1-[2-carboxyphenylazo]-2-naphthol (CPAN) and 1-[2-nitrophenylazo]-2-naphthol (NPAN). The antimicrobial activities of the synthesized azo compounds were reported invitro using disc diffusion method.

EXPERIMENTAL

All chemicals used in the present investigation were of analytical grade. 2-naphthol and dimethylformamide were purchased from Sigma-Aldrich.

Elemental analysis (C, H, N) of azo compounds were performed at Central Drug Research Institute, Lucknow. The purity of the synthesized compounds were checked by TLC using the standard Biochem Kit, Madurai (India). The melting points were determined in open capillary tubes using Prefit model. The molecular weight was determined by cryoscopic method using glacial acetic acid as solvent. In the present investigation the IR spectra of azo compounds were recorded on Schimadzu FTIR spectrophotometer model 8400S in KBr wafer and the NMR spectra were obtained on JEOL AL 300, 300.4 MHz FT NMR spectrometer using $CDCl_3$ as solvent and reported relative to TMS as internal standard.

Synthesis of azo compounds

Azo compounds were synthesized according to the method reported in literature [25]. There are two steps in the synthesis of azo compounds:

Step I : Diazotisation

A mixture of freshly distilled amine (0.016 mol) and concentrated hydrochloric acid was stirred until a clear solution was obtained. This solution was cooled to 0–5 ºC, and a solution of sodium nitrite in 10mL water was then added dropwise, maintaining the temperature below 5 º C. The resulting mixture was stirred for an additional 30 min in an ice bath and was then buffered (pH ~ 5.0) with solid sodium acetate trihydrate .

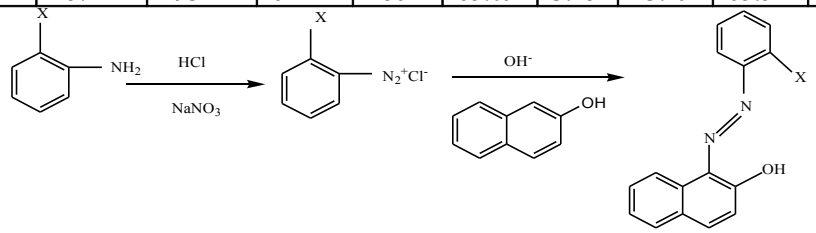
Step II: Coupling

2-Naphthol (0.016 mol) was dissolved in 8ml 10% sodium hydroxide, and cooled to 0–5 ºC in an ice bath. This solution was then gradually added to the cooled benzenediazonium chloride solution, and the resulting mixture was stirred at 0–5°C for 60 min. The resulting crude precipitate was filtered, washed several times with cold water and was recrystallized from hot chloroform to yield azo compound.

Azo compounds were synthesized according to following scheme 1. The physical and analytical data obtained for these compounds are shown in Table 1.

Table 1: Physical and Analytical data of substituted azo compounds

Compound	Molecular formula	Molecular weight		Yield %	M.P. °C	Elemental analysis						R_f
						% Found			% Calculated			
		Found	Calcd.			C	H	N	C	H	N	
I (PAN)	$C_{16}H_{12}N_2O$	244	252	67	260	76.56	5.09	10.72	77.10	5.22	11.24	76.32
II (HPAN)	$C_{16}H_{12}N_2O_2$	261	267	79	265	71.91	3.42	9.84	72.45	4.90	10.56	67.08
III MPAN)	$C_{17}H_{14}N_2O_2$	276	280	75	272	72.21	4.07	9.89	73.38	5.03	10.07	53.83
IV ClPAN)	$C_{16}H_{11}N_2OCl$	277	287	64	279	66.97	3.31	8.76	68.08	3.90	9.92	62.09
V (CPAN)	$C_{17}H_{12}N_2O_3$	285	292	73	283	68.21	3.69	7.90	69.86	4.10	9.58	55.45
VI (NPAN)	$C_{16}H_{11}N_3O_3$	287	293	61	286	65.09	3.45	13.29	65.52	3.75	14.33	53.10



I X = H

II X = OH

III X = OCH_3

IV X = Cl

V X = COOH

IV X = NO_2

Scheme 1 Preparative route of substituted azo compounds

Antimicrobial activity

The synthesized azo compounds were screened for the presence of antibacterial constituents against six strains of bacteria i.e. *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa, Enterobacter cloacae*, *Enterococcus faecalis* and one species of fungi i.e. against *Candida albicans* by disc diffusion method [26, 27]. Nutrient agar was used as culture medium for bacterial growth while fungi were subcultured in potato dextrose agar medium. Measured quantities of the test compounds were dissolved in DMF to get final concentrations of 500 ppm and soaked in filter paper discs of 6 mm diameter. These discs were placed on the previously seeded plates and incubated at 35^{0}C. All compounds were dissolved in DMF. Ciprofloxacin (5 mcg/disc for bacteria) and ketoconozole (100 units/disc for fungi) was used as reference antibiotic and DMF as control. The zones of inhibition were determined at the end of an incubation period of 24 hr at 37° C. During this period, the test solution diffused and the growth of inoculated microorganism was affected. The bacterial inhibition zone values are summarized in Table 4 and its statistical presentation is shown in fig 1.

Minimum Inhibitory Concentration (MIC)

The minimal inhibitory concentration (MIC) was determined by broth microdilution method [28]. For MIC determination, the inoculums was prepared using a 4.6 hr broth culture of each bacterial strains adjusted to a turbidity equivalent to a 0.5 Mc Farland standard, diluted in Nutrient broth media to give concentration of ≈106 cfu/mL for bacteria. Twofold serial dilutions of compounds were prepared in Nutrient broth in 96-well plates starting from a stock solution of compounds (2.00 mg/mL DMF). DMF had no effect on the microorganism in the concentrations studied. An equal volume of bacterial inoculum was added to each well on the microtitre plate. In this manner final concentration of compounds range 500-0.49 µg/mL and 5x105 cfu/mL bacteria in each well (last wells are broth only control well). The inoculated microtiter plates were then incubated at 37°C for 24 h, and the growth was recorded spectrophotometrically at 620 nm using a microplate reader. The MIC value was defined as the lowest concentration of compounds whose absorbance was comparable with the negative control wells (broth only, without inoculum). The MIC values are reported at Table 5 as the mean of three replicates.

RESULTS AND DISCUSSION

In this study, azo compounds were synthesized according to the preparative route illustrated in Figure 1.

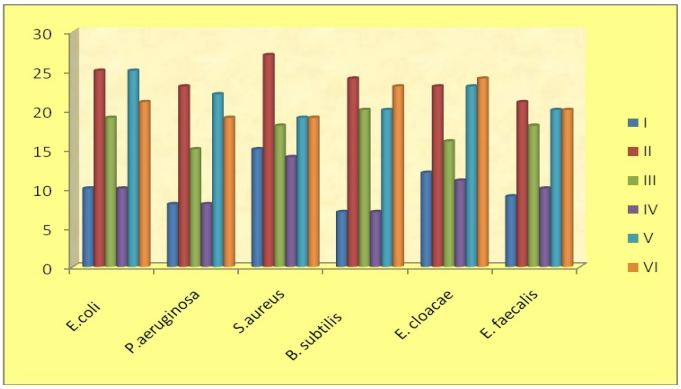


Fig 1 Statistical representation for biological activity of azo compounds.

Table 2: Infrared spectral data of substituted azo compound

Compound	X	ν_{O-H} (cm^{-1})	ν_{N-H} (cm^{-1})	$\nu_{C=C}$ in plane skeletal vibrations (cm^{-1})			$\nu_{N=N}$ (cm^{-1})	ν_{C-N} (cm^{-1})	ν_{C-O} (cm^{-1})
I	H	3310	2810	1600	1530	1480	1440	1320	1140
II	OH	3300	2790	1580	1520	1470	1430	1310	1130
III	OCH_3	3310	2810	1580	1530	1470	1445	1330	1130
IV	Cl	3310	2820	1600	1530	1480	1450	1330	1140
V	COOH	3300	2790	1580	1520	1470	1430	1310	1130
VI	NO_2	3320	2830	1610	1540	1480	1460	1340	1150

Spectroscopic characterization of synthesized compounds

Infrared spectra: The glance at the structure of azo compounds, one may expect the absorption bands due to O-H, N=N, -N-H, C=C, =C-H, C-N and C-O vibrations in IR region. Important IR peak values of azo compounds are given in Table 2. Apperance of -N-H stretching vibration in the IR spectrum of azo compounds can be attributed to –O-H···N tautomeric shift and intramolecular hydrogen bonding [29, 30] in the compounds under investigation. The data in table 2 shows that the compounds which can form stronger hydrogen bonds with nitrogen atom (X= OH and COOH) absorbs at lower frequencies than the compounds which forms weaker hydrogen bonds (X= Cl and OCH_3) because OH and COOH by forming stronger hydrogen bonds can extend conjugation for whole system so favors a lower N=N and C=C bond order hence shift of these bonds towards lower wave number.

Nuclear Magnetic Resonance Spectra

^{1}H NMR spectra

The ^{1}H NMR spectra of azo compounds under investigation show the characteristic signals due to following protons.

Naphthyl O-H protons

All azo compounds show singlets in the region 11.50-11.65 ppm. Such a downfield absorption shows the strong intramolecular hydrogen bonding between the hydroxyl proton and azo nitrogen.

Aryl protons

Signals for naphthyl and phenyl =C-H can be seen in the ^{1}H NMR spectra of all azo compounds in the region 7.11- 8.40 ppm. The position of these signals shifts according to the substituent present on phenyl and naphthyl rings. Table 3 shows the effect of electron donating and electron withdrawing groups on the position of signals.

Hydrazone protons

As discussed above azo compounds exhibit azo/hydrazone tautomerism so signals for N-H protons appear in the downfield region (9-11ppm) for all azo compounds indicating the strong intramolecular hydrogen bonding.

^{1} H NMR spectral data of the synthesized azo compounds is shown in Table 3.

^{13}C NMR spectra

Azo compounds exhibit absorption signals due to the carbon atom of naphthyl and phenyl groups. The signals for naphthylic carbon atoms were seen in the range of 117 – 146.2 ppm. The absorption signals for naphthylic carbon atoms have not shown larger shifts by substitution at ortho position of phenyl rings. In examining the signals of phenyl carbon atoms signals were seen in the range of 112.5 – 154.5 ppm. The absorption signals for these carbon atoms does shifts towards upfield region due to the presence of electron releasing substituent while a shift towards downfield region is observed due to the presence of electron withdrawing substituent.

Table 3: ^{1}H NMR spectral data of substituted azo compounds

Compound No.	X	δppm	Hydrogen	Multiplicity	Assignment
I	H	11.56 7.14-7.80	1 11	Singlet Multiplet	O-H protons (naphthyl) Ar-H protons
II	OH	11.50 7.13-7.80 10.40	1 10 1	Singlet Multiplet Singlet	O-H protons (naphthyl) Ar-H protons O-H protons (phenyl)
III	OCH_3	11.55 7.11-7.80 3.75	1 10 3	Singlet Multiplet Singlet	O-H protons (naphthyl) Ar-H protons Methoxy protons
IV	Cl	11.63 7.17-7.89	1 10	Singlet Multiplet	O-H protons (naphthyl) Ar-H protons
V	COOH	11.61 7.15-8.31 12.83	1 10 1	Singlet Multiplet Singlet	O-H protons (naphthyl) Ar-H protons Carboxylic protons
VI	NO_2	11.65 7.18-8.40	1 10	Singlet Multiplet	O-H protons (naphthyl) Ar-H protons

1-[2-Methoxyphenylazo]-2-naphthol and 1-[2-Carboxyphenylazo]-2-naphthol show absorption signals for methyl protons and carboxylic protons in the region 56.6 ppm and 170 ppm respectively.

Antimicrobial activity

Bactericidal and fungicidal activities of the synthesized azo compounds against pathogenic bacteria and fungi were recorded by disc diffusion method and results given in Table 4. All the azo compounds showed remarkable activity against used microbs and results were compared with standard drugs. From the results (Table 4), it is concluded that insertion of substituent at the ortho position of PAN (I) increases its antimicrobial activity . HPAN (I) and CPAN (V) exhibited wide spectrum of activity, as they were highly active against all cultures but *o*-chloride substituent involved in the III compound (ClPAN) do not modify the antibacterial effect of I compound (PAN). This phenomenon could be because the *o*-chloride is an electron withdrawing group inductively but possibly it can act as an electron donating group through resonance on phenyl ring and by this does not affect the antibacterial activity of PAN (I). MPAN (III) and NPAN (VI) also showed enhances activity than PAN (I).

Table 4 Antimicrobial screening data (zone of inhibition in mm) of the synthesized azo compounds.

Compounds	*E.coli* (Gram negative)	*P.aeruginosa* (Gram negative)	*S.aureus* (Gram positive)	*B. subtilis* (Gram positive)	*E. cloacae* (Gram positive)	*E. faecalis* (Gram positive)	*C. albicans*
I (PAN)	10	8	15	7	12	9	-
II (HPAN)	25	23	27	24	23	21	11
III (MPAN)	19	15	18	20	16	18	8
IV (ClPAN)	10	8	14	7	11	10	-
V (CPAN)	25	22	19	20	23	20	13
VI (NPAN)	21	19	19	23	24	20	10
Standard	22	19	25	21	22	19	18

The results of fungicidal screening (Table 4) shows that among all the tested azo compounds HPAN (I) and CPAN (V) were highly active, MPAN (III) and NPAN (VI) were moderately active while PAN (I) and ClPAN (IV) were inactive against fungal species. The variation in the effectiveness of different compounds against different organisms depends either on the impermeability of the cells of the microbes or on differences in ribosome of microbial cells [31].

Mode of action. Although the exact mechanism is not understood biochemically, mode of action of antimicrobials may involve various targets in microorganisms [32].

(i) Interference with the cell wall synthesis, damage as a result of which cell permeability may be altered (or) they may disorganize the lipoprotein leading to the cell death.

(ii) Deactivate various cellular enzymes, which play a vital role in different metabolic pathways of these microorganisms.

(iii) Denaturation of one or more proteins of the cell, as a result of which the normal cellular processes are impaired.

(iv) Formation of a hydrogen bond through the azo group with the active centre of cell constituents, resulting in interference with the normal cell process.

Minimum inhibitory concentration (MIC)

MIC is the lowest amount of drug at which it is able to inhibit the growth of specified microorganism. MIC value of the synthesized azo compounds were calculated against two strains of gram positive (*S. aureus* and *B. subtilis*) and two strain of gram negative bacteria (*E. coli* and *P. aeruginosa*) using broath microdilution method. The comparison of MIC values (Table 5) of all six azo compounds indicates that the –OH and –COOH groups as substituents on the phenyl ring causes a substantial increase in antimicrobial activity. Table 5 reveals that the presence of a chloro substituent group on the phenyl rings contributes almost nothing to the antimicrobial activity. The general trend for activity on the basis of MIC values in decreasing order can be given as: II > V > VI > III > IV ~ I.

Table 5 Minimum inhibitory concentrations (MIC) in µg/mL of azo compounds.

Compounds	E. coli (Gram negative)	P. aeruginosa (Gram negative)	S. aureus (Gram positive)	B. subtilis (Gram positive)
I (PAN)	65.0	75.0	75.0	80.0
II (HPAN)	40.0	47.0	38.0	35.0
III (MPAN)	35.0	40.0	45.0	50.0
IV (ClPAN)	65.0	75.0	75.0	80.0
V (CPAN)	46.0	50.0	57.0	62.0
VI (NPAN)	55.0	55.0	67.0	65.0

Conclusions

Six azo compounds have been prepared and characterized on the basis of analytical and spectral data. Screening of these compounds against pathogenic microorganism reveals that these compounds have the capacity of inhibiting metabolic growth of *some microorganisms* to different extent. The antimicrobial activity of the compounds depends on the nature of substituent present on the aromatic ring.

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