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Synthesis and *in vitro* antibacterial evaluation of 2,4-dintrophenol incorporated azo dyes molecules

Ramesh F. Pagariya[1] and Nandkishor S. Thakre[2]

[1]Department of Chemistry, R. A. Arts, M. K. Commerce and Shri. S. R. Rathi Science College, Washim, India
[2]M. S. Patil Arts, Science and Late P. T. Commerce College, Manora, Dist-Washim

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ABSTRACT

Synthesis of most azo dyes involves diazotization of a primary aromatic amine, followed by coupling with one or more nucleophiles. Thus, benzoic, phenolic, salicylic and naphtholic compounds acts as nucleophiles and undergoes coupling reactions. In this study, a series of azo compounds were synthesized via the diazotization of different aromatic amines followed by coupling with 2, 4, dinitrophenol. These compounds were characterized by elemental analysis, IR, [1]HNMR and MASS spectroscopic techniques. The synthesized compounds have been tested in vitro against human pathogens in order to assess their antibacterial properties using disk diffusion method. The compounds analysed for its antibacterial action showed moderate to significant inhibitory effect at some specific concentrations against the tested pathogens.

Keywords: 2, 4-dinitrophenol, Azo compounds, Antibacterial Activity, Human pathogens

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INTRODUCTION

Azo compounds are the most fundamental class of commercial dyes and are well coloured that have been used as dyes and pigments [1-2]. Organic colour chemistry is undergoing very exciting development as a result of the opportunities presented by dye applications in science and technology fields such as, pharmaceutical, cosmetic, textile and leather industries, electronic devices, linear and non-linear optics [3-7]. It has been found that, existence of an azo (-N=N-) moiety in different types of azo derivatives has caused them to show antibacterial and pesticidal activity [8-9]. Hence in the present time, exploration of azo dye as antibacterial and antifungal agents has received considerable attention [10-12].

For the reason of variety applications of azo compounds, it is interesting to study synthesis of such new azo compounds and their derivatives in order to explore the newer potentials of such compounds. Several azo compounds derived from thymol [13], aspirin [14], paracetamol [15], m-cresol [16], resorcinol [17] and vanillin [18] moieties have been frequently reported and exhibited excellent biological properties.

In view of the above relevant literature survey, it can be said that, compounds with azo moiety have been extensively used as dyes due to their utility in colorings functions. But their variety in biological activity potential is less reported and as a consequence still have a huge scope to synthesize new azo compounds and to test their biological activities. So, in the present work, we have synthesized eight new azo compounds namely 3a to 3h containing 2, 4-Dinitrophenol moiety and characterized by FTIR, [1]HNMR and MASS spectral technique. The antibacterial activities of the synthesized azo compounds were reported in vitro using disc diffusion method.

MATERIALS AND METHODS

The chemicals used in the present studies are of synthetic grade, Merck company ltd. The products were

characterized by IR, ^{1}HNMR and MASS spectral studies. The M.Ps. were determined by open capillary method using digital melting point apparatus model 935/934 by Electronics India and is uncorrected. The IR spectra were recorded on FTIR Spectrophotometer Model RZX (Perkin-Elmer) in the form of KBr pallet. ^{1}HNMR spectra were recorded in $CDCl_3$ on a FT-NMR Cryomagnet Spectrometer 400 MHz (Bruker) using TMS as an internal standard and MASS spectra were recorded on LC-MS Spectrometer Model Q-ToF Micro Waters. The purity of compounds was checked by TLC. The crude products were recrystallised from 75% ethanol.

General procedure for synthesis of azo compounds [19-21]

Substituted aromatic amines (0.01mole) were mixed with 2.5 ml conc. HCl and 2.5 ml (4N) cold solution of $NaNO_2$ was added with the stirring. The temperature of the reaction was maintained up to 0-5^0 C. Diazonium salt solution so prepared was added drop wise to the alkaline 10% NaOH solution of 2, 4 dinitrophenol (0.01mole). The reaction mixture was stirred for 30-45 minutes maintaining the temperature 5-10^0 C. The coloured products obtained was filtered, washed with water and recrystallized from 75 % ethanol. The general reaction scheme for synthesis of azo compounds of 2, 4 dinitrophenol is shown in figure-(1). Also code, chemical name, molecular formulae, molecular weights, melting points and percentage yield of synthesized azo compounds of 2, 4 dinitrophenol is shown in table-(1).

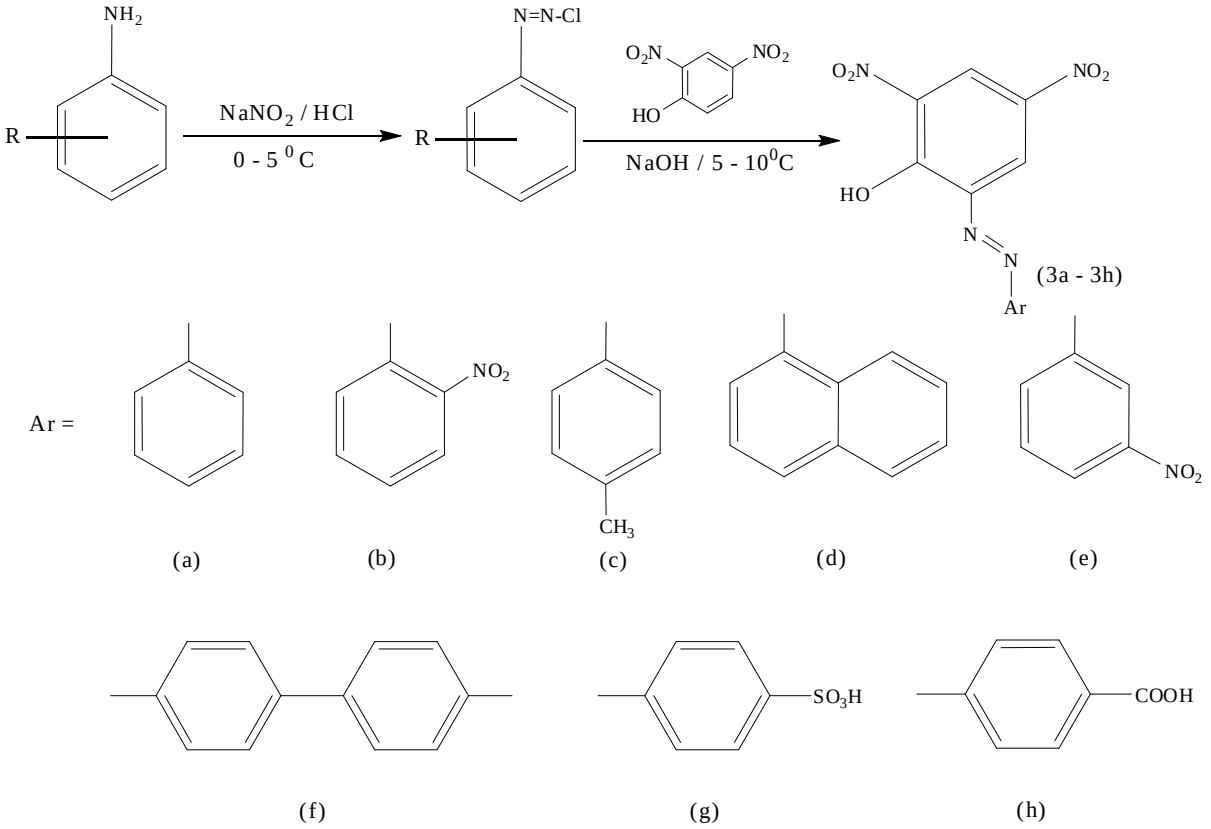

Figure-(1) The general reaction scheme for synthesis of azo compounds of 2, 4-Dinitraophenol

Table-(1) The code, name, molecular formulae, molecular weights, melting points and percentage yield of synthesized azo compounds of 2,4-Dinitrophenol

Code	Compound Name	Molecular Formulae	Mol. Wt.	M.P ^{0}C	% Yield
3a	(*E*)-2,4-dinitro-6-(phenyldiazenyl) phenol	$C_{12}H_8N_4O_5$	288.22	090	61
3b	(*E*)-2,4-dinitro-6-[(2-nitrophenyl)diazenyl)] phenol	$C_{12}H_7N_5O_7$	333.21	118	62
3c	(*E*)-2,4-dinitro-6-(*p*-tolyldiazenyl)] phenol	$C_{13}H_{10}N_4O_3$	302.24	106	56
3d	(*E*)-2-(naphthalen-1-yldiazenyl)-4,6-dinitro phenol	$C_{16}H_{10}N_4O_5$	338.27	095	58
3e	(*E*)-2,4-dinitro-6-[(3-nitrophenyl)diazenyl)] phenol	$C_{12}H_7N_5O_7$	333.21	160	60
3f	6,6'-(1E,1E')-biphenyl-4,4'-diylbis(diazene-2,1diyl) bis(2,4–dinitrophenol)	$C_{24}H_{14}N_8O_{10}$	574.42	116	56
3g	(*E*)-4-[2-hydroxy-3,5 dinitrophenol) diazenyl] benzenesulfonic acid	$C_{12}H_8N_4O_8S$	368.28	110	63
3h	(*E*)-4-[(2-hydroxy-3, 5-dinitrophenyl) diazenyl] benzoic acid.	$C_{13}H_8N_4O_7$	332.23	098	69

Antimicrobial activity:

The compounds 3a-3h were screened for the presence of antibacterial constituents against four microorganisms viz.,

Escherichia coli, *Staphylococcus aureus*, *Pseudomonas aeroginosa* and *Salmonella typhi*, adopting disc diffusion method [22-23]. All the bacterial cultures were obtained from NCL reference laboratory, Pune. The compounds were dissolved in ethanol to give 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/mL solutions. Sterile discs were dipped in solutions, dried and placed on nutrient agar plates spread with the bacteria. The plates were further incubated for 24 hrs at 37^0C and the zones of inhibition were measured using antibiotic zone reader (Hi- Media).

RESULTS AND DISCUSSION

Spectroscopic study:

I.R., ^{1}HNMR and MASS spectra showed the expected signals / peaks which correspond to various groups present in each compounds. The I.R. ^{1}HNMR and MASS spectral data is shown in Table (2).

Table (2):- IR, ^{1}HNMR and Mass Spectral Data

Compound	Spectra	IR, ^{1}HNMR AND MASS SPECTROSCOPIC DATA/PEAKS
3a	IR (KBr, cm^{-1}) ^{1}HNMR(δppm) MASS (m/z,%)	3581 (Phenolic -OH stretch), 3093 (C-H stretch), 1601 (C=C stretch), 1481 (N=N Stretch), 1346 (-NO_2 stretch), 1284 (C-N stretch), 1257 (C-O stretch). 4.85 (s 1H of -OH), 6.90 to 8.72 (m 7H of Ar-H). LC-MS (m/z, %): 287.0 (M^+, 27.9), 183.0 ($O_2NO_2NC_6H_2OH$, 100), 105.1 (C_6H_5N=N, 36.9), 91.5 (C_6H_2OH, 7.0%).
3b	IR (KBr, cm^{-1}) ^{1}HNMR(δppm) MASS (m/z,%)	3269 (Phenolic -OH stretch), 3077 (C-H stretch), 1599 (C=C stretch), 1479 (N=N Stretch), 1347 (C-N stretch), 1333 (-NO_2 stretch), 1256 (C-O) stretch) 4.94 (s 1H of –OH), δ 6.92 to 9.07 (m 6H of Ar-H) LC-MS (m/z, %): 332.2 (M^+, 16.9), 183.0 ($O_2NO_2NC_6H_2OH$, 100), 150.1 ($O_2NC_6H_4N$=N, 36.9), 91.1 (C_6H_2OH, 11.0%).
3c	IR (KBr, cm^{-1}) ^{1}HNMR(δppm) MASS (m/z,%)	3583 (Phenolic -OH stretch), 3095 (C-H stretch), 1603 (C=C stretch), 1485 (N=N Stretch), 1344 (C-N) stretch), 1285 (-NO_2) stretch), 1145 (C-O stretch), 835 (Ar-C-C stretch). 2.43 (s 3H of -CH_3), 4.84 (s 1H of -OH), 6.94 to 8.20 (m 6H of Ar-H) LC-MS (m/z, %): 301.2 (M^+, 36.6), 287.0 ($O_2NO_2NOHC_6H_2$ N=NC_6H_4, 3.6), 183.0 ($O_2NO_2NOHC_6H_2$, 100), 104.1 (C_6H_4 N=N, 35.9), 91.1 (C_6H_2OH, 10.9%).
3d	IR (KBr, cm^{-1}) ^{1}HNMR(δppm) MASS (m/z,%)	3450 (Phenolic -OH stretch), 3109 (C-H stretch), 1598 (C=C stretch), 1434 (N=N Stretch), 1346 (C-N) stretch), 1257 (-NO_2) stretch), 1138 (C-O stretch). 2.43 (s 3H of -CH_3), 4.84 (s 1H of -OH), 6.94 to 8.20 (m 6H of Ar-H) LC-MS (m/z, %): 337.2 (M^+, 26.9 2d), 183.0 (O_2NO_2N OHC_6H_2, 100), 153.0 ($C_{10}H_7N$=N, 11.5), 91.1 (C_6H_2OH, 10.5).
3e	IR (KBr, cm^{-1}) ^{1}HNMR(δppm) MASS (m/z,%)	3270 (Phenolic -OH stretch), 3109 (C-H stretch), 1598 (C=C stretch), 1433 (N=N Stretch), 1334 (C-N) stretch), 1256 (-NO_2) stretch), 1107 (C-O stretch) 5.16 (s 1H of -OH), δ 6.92 to 9.08 (m 6H of Ar-H) LC-MS (m/z, %): 332.2 (M^+, 16.5), 183.0 ($O_2NO_2NOHC_6H_2$, 100), 150.1 ($O_2NC_6H_4N$=N, 36.5), 91.1 (C_6H_2OH, 10.3).
3f	IR (KBr, cm^{-1}) ^{1}HNMR(δppm) MASS (m/z,%)	3523 (Phenolic -OH stretch), 3271 (N-H stretch), 3109 (C-H stretch), 1599 (C=C stretch), 1434 (N=N Stretch), 1347 (C-N) stretch), 1256 (-NO_2) stretch), 1109 (C-O stretch). 5.37 (s 1H of –OH), 6.94 to 9.92 (m 12H of Ar-H) LC-MS: (m/z, %): 573.1(M^+, 6.4), 390.0 ($O_2NO_2NOHC_6H_2$ N=$NC_6H_4C_6H_4N$=N, 4.3), 208.0 (N=$NC_6H_4C_6H_4N$=N, 6.0), 183.0 ($O_2NO_2NOHC_6H_2$, 100), 153.0 ($C_6H_4C_6H_4$, 11.8)
3g	IR (KBr, cm^{-1}) ^{1}HNMR(δppm) MASS (m/z,%)	3555 cm^{-1}(Phenolic -OH stretch), 3269 (-OH of SO_3H stretch), 3109 (C-H stretch), 1598 (C=C stretch), 1433 (N=N Stretch), 1347 (-NO_2 stretch), 1255 (C-N) stretch), 1108 (C-O) stretch), 743 (C-S stretch). 4.99 (s 1H of -OH), 7.27 to 9.07 (m 6H of Ar-H), 11.03 (s 1H of -SO_3H) LC-MS (m/z, %): 367.1 (M^+, 22.0), 184.0 ($HO_3SC_6H_4N$=N, 13.5), 183.0 ($O_2NO_2NOHC_6H_2$, 100), 91.1 (C_6H_2OH, 10.2).
3h	IR (KBr, cm^{-1}) ^{1}HNMR(δppm) MASS (m/z,%)	3582 (Acidic -OH stretch), 3524 (Phenolic -OH stretch), 3094 (C-H stretch), 1602 (C=O stretch), 1531 (C=C stretch), 1435 (N=N Stretch), 1345 (-NO_2 stretch), 1256 (C-N stretch), 1144 (C-O stretch). 4.81 (s 1H of -OH), 6.88 to 8.73 (m 6H of Ar-H), 12.59 (s 1H of -COOH). LC-MS (m/z, %): 331.2 (M^+, 16.9), 183.0 ($O_2NO_2NOHC_6H_2$, 100), 149.1 (HOOCOH C_6H_4N=N, 8.0).

A total eight azo compounds of 2, 4-Dinitrophenol have been synthesized, recrystallised and six different concentrations of each compound were prepared and further used individually to analyze its antibacterial activity against four human pathogens viz. *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeroginosa* and *Salmonella typhi.* The data on antimicrobial activity of azo compounds of 2, 4-Dinitrophenol 3a-3h against four human pathogens are presented in table-(3) to table-(6). From the results it was observed that the azo compounds of 2, 4-Dinitrophenol have showed moderate to significant antibacterial potential against all four pathogens.

Antibacterial properties of the synthesized azo compounds of 2, 4-Dinitrophenol viz 3a-3h [Zone of inhibition (mm)]

Table (3):- Effect of azo compounds of 2, 4-Dinitrophenol viz. 3a-3h on the growth response of *Escherichia coli*

Conc. (mg/mL)	3a	3b	3c	3d	3e	3f	3g	3h
0.5	I (10)	I (10)	NI	I (12)	I (10)	NI	I (10)	NI
1.0	I (10)	NI	I (11)	I (12)	1.0	NI	NI	NI
1.5	NI	NI	I (11)	NI	NI	NI	NI	I (10)
2.0	I (10)	NI	NI	I (12)	I (10)	I (10)	NI	I (10)
2.5	I (10)	NI	NI	I (11)	NI	I (10)	I (11)	I (10)
3.0	I (10)	I (10)	I (10)	I (12)	I (10)	I (10)	I (10)	I (12)

I = *Inhibition, values of inhibition are given in parenthesis,* NI = *Not inhibition*

Table (4):-Effect of azo compounds of 2, 4-Dinitrophenol viz. 3a-3h on the growth response of *Staphylococcus aureus*

Conc. (mg/mL)	3a	3b	3c	3d	3e	3f	3g	3h
0.5	I (10)	NI	NI	I (14)	I (11)	NI	NI	NI
1.0	I (10)	NI	I (11)	I (10)	I (11)	NI	NI	NI
1.5	NI	NI	I (11)	I (10)	I (10)	I (10)	NI	I (10)
2.0	NI	NI	NI	NI	I (11)	NI	I (10)	NI
2.5	NI	NI	NI	I (10)	I (10)	NI	NI	NI
3.0	I (10)	I (10)	NI	I (11)	I (10)	NI	NI	NI

I = *Inhibition, values of inhibition are given in parenthesis,* NI = *Not inhibition*

Table (5):-Effect of azo compounds of 2, 4-Dinitrophenol viz. 3a-3h on the growth response of *Pseudomonas aeroginosa*

Conc. (mg/mL)	3a	3b	3c	3d	3e	3f	3g	3h
0.5	NI	NI	NI	I (10)	I (10)	NI	NI	I (10)
1.0	NI	NI	I (10)	I (10)	NI	NI	NI	NI
1.5	NI	NI	NI	I (10)	NI	NI	NI	NI
2.0	NI	NI	NI	NI	I (10)	I (10)	NI	I (12)
2.5	NI	NI	I (11)	I (12)	I (10)	I (10)	NI	NI
3.0	NI	NI	I (10)	NI	I (11)	I (10)	NI	I (12)

I = *Inhibition, values of inhibition are given in parenthesis,* NI = *Not inhibition*

Table (6):-Effect of azo compounds of 2, 4-Dinitrophenol viz. 3a-3h on the growth response of *Salmonella typhi*

Conc. (mg/mL)	3a	3b	3c	3d	3e	3f	3g	3h
0.5	NI	NI	NI	NI	I (12)	NI	NI	NI
1.0	NI	NI	I (12)	NI	I (10)	NI	NI	NI
1.5	I (16)	NI	NI	NI	I (10)	NI	NI	NI
2.0	I (16)	NI	NI	NI	I (12)	I (10)	NI	I (10)
2.5	NI	NI	NI	NI	I (10)	I (10)	NI	NI
3.0	I (10)	NI	NI	NI	I (12)	NI	NI	NI)

I = *Inhibition, values of inhibition are given in parenthesis,* NI = *Not inhibition*

The results regarding antibacterial activity of eight azo compounds of 2, 4-dinitrophenol viz 3a–3h against *E.Coli* are presented in table (3) and figure (2). The maximum antibacterial activity was observed in case of derivative 3d and 3h for which nearly all concentrations used were showed significant antibacterial effect against *E.Coli* and the average diameter of zone of inhibition ranges from 10–12 mm. This is followed by 3a, 3c, 3d, 3f, and 3g derivatives for which most of the different concentrations showed moderate antibacterial effect with average diameter of zone of inhibition ranges from 10–11 mm recorded over control where antibacterial activity was not observed.

The results on antibacterial activity of eight azo compounds of 2, 4-dinitrophenol viz 3a– 3h against *S.aureus* are tabulated in table (4) and figure (3). From the result it was observed that the compounds 3d and 3e showed pronounced antibacterial activity at nearly all the six different concentrations except at 2.5 mg/mL for 3d. The peak zone of inhibition recorded 14 mm diameter at 0.5 mg/mL for 3d over control. This is followed by 3a derivative that have exhibited moderate antimicrobial activity at 0.5, 1.0, 3.0 mg/mL concentration with zone of inhibition recorded 10 mm diameter and 3c derivative showed moderate antimicrobial activity merely at 1.0, 1.5 mg/mL with maximum zone of inhibition recorded 11 mm against *S.aureus*. The compounds 3b, 3f, 3g and 3h were found scarce to inhibit the growth of *S.aureus* species

The antibacterial effect of eight azo compounds viz 3a–3h against *Pseudomonas aeroginosa* species are recorded in table (5) and figure (4). From the results it was observed that the azo derivative 3c, 3d, 3e, 3f and 3g showed moderate antibacterial effect against *Pseudomonas* species at three to four different concentrations used with average zone of inhibition ranging from 10–12 mm diameter with maximum zone of inhibition 12 mm at 2.5 mg/mL for 3d and 2.0, 3.0 mg/mL for 3h over control. However a compound 3a, 3b and 3g were found unsuccessful to

inhibit the growth of *Pseudomonas aeroginosa.*

The pursuit of data on antimicrobial effect of azo compounds viz 3a–3h against *Salmonella typhi* is shown in table (6) and figure (5). The maximum antibacterial activity was recorded at all the six different concentrations in derivative 3e and at three different concentration in derivative 3a where average zone of inhibition ranges from 10–16 mm with maximum zone of inhibition 16 mm recorded at 1.5, 2.0 and 3.0 mg/mL for 3a. This is followed by 3c and 3f which showed antibacterial effect at only one or two different concentrations respectively with average zone of inhibition ranging from 10–12 mm with maximum zone of inhibition 12 mm against *S. typhi* at 1.0 mg/mL for 3c over control.

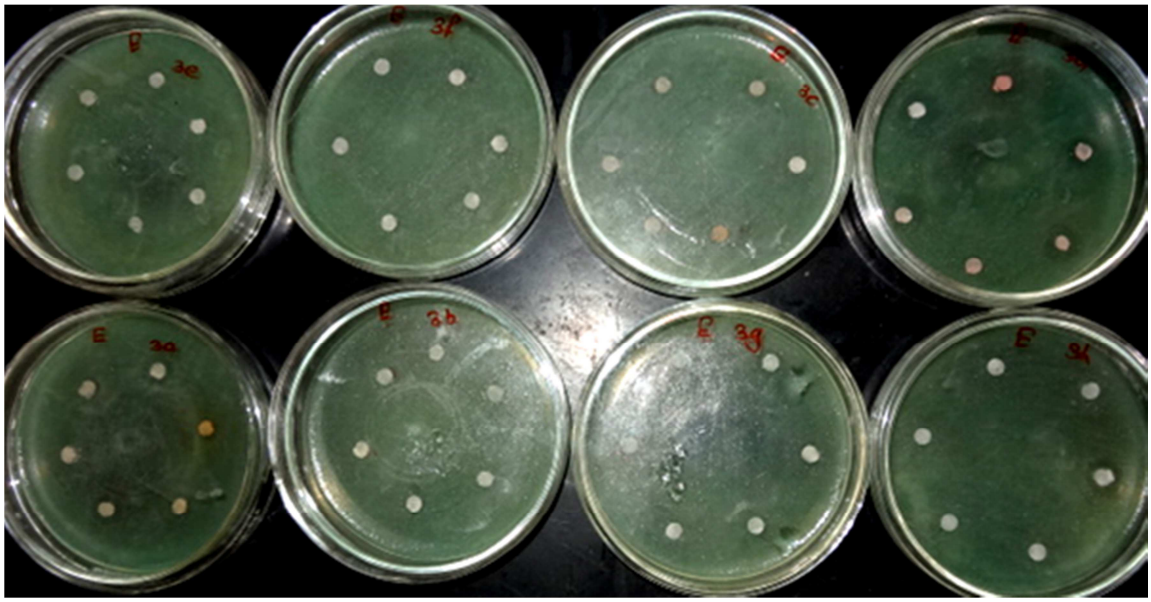

Figure 2: Effect of azo compounds of 2, 4-dinitrophenol viz. 3a–3h on the growth of *E.Coli*

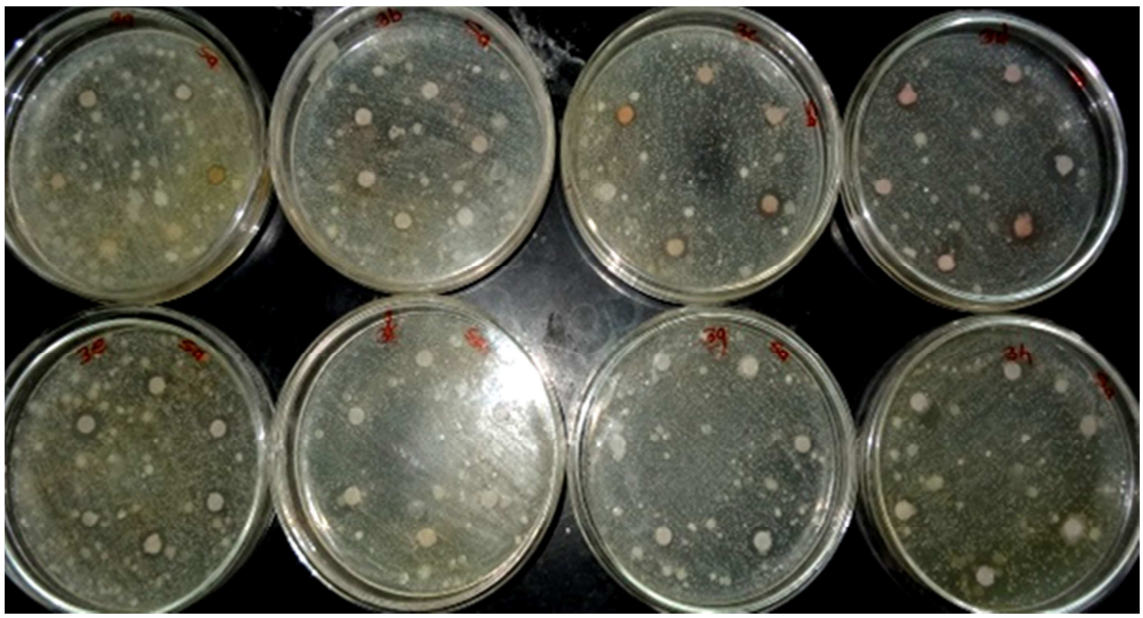

Figure 3: Effect of azo compounds of 2, 4-dinitrophenol viz. 3a–3h on the growth of *Staphylococcus aureus*

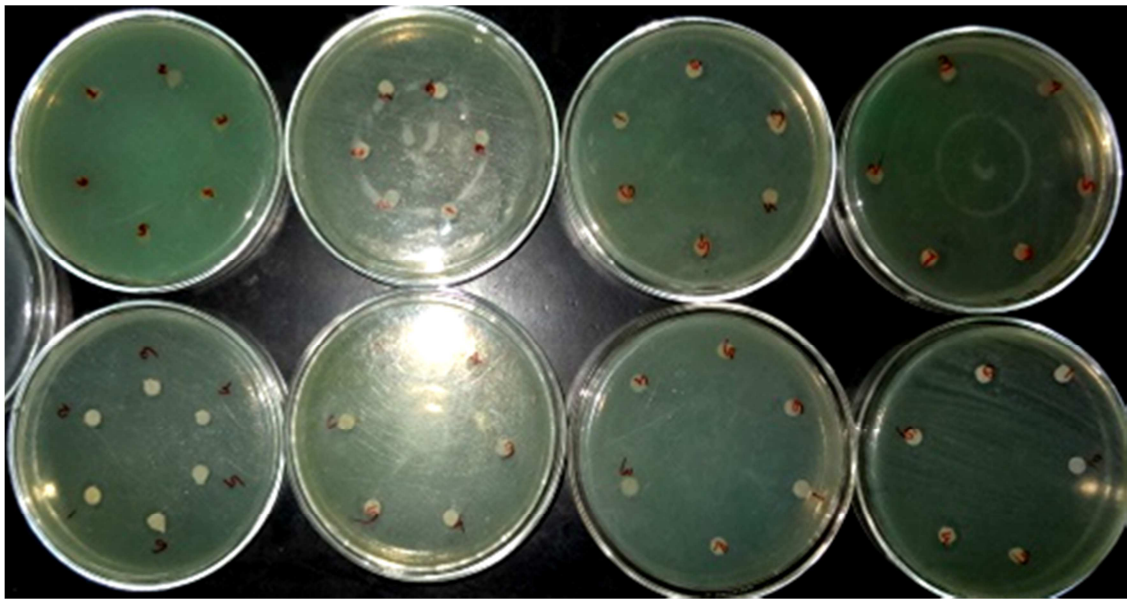

Figure 4: Effect of azo compounds of 2, 4-dinitrophenol viz. 3a–3h on the growth of *Pseudomonas aeroginosa*

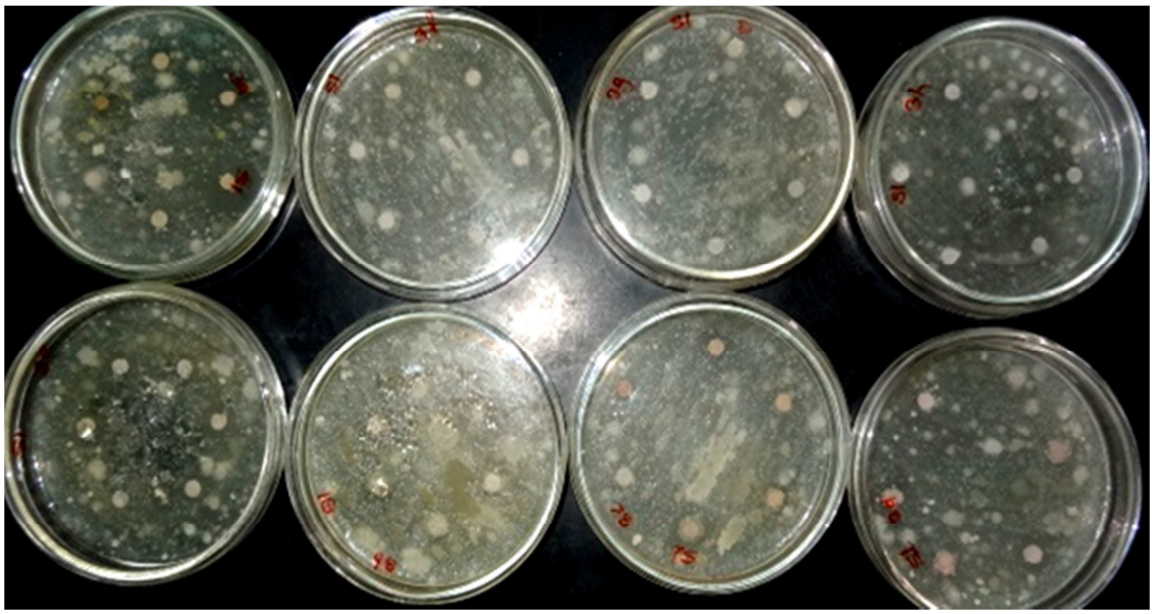

Figure5: Effect of azo compounds of 2, 4-dinitrophenol viz. 3a–3h on the growth of *Salmonella typhi*

CONCLUSION

All the eight novel azo compounds 3a–3h containing 2,4-dinitrophenol moiety were successfully synthesized in average yield and their structures are elucidated using elemental analysis, FTIR, ^{1}HNMR & MASS spectroscopy.

The results on antibacterial evaluation study reveals that all the eight newly synthesized compounds viz 3a–3h found to have moderate to significant antibacterial effect against *E.Coli*, *S. aureus*, *Pseudomonas aeroginosa*, and *Salmonella typhi* at two or more different concentrations analysed. Among the eight azo derivatives, 3d is extraordinarily effective against *E.Coli*, *S. aureus*, *Pseudomonas aeroginosa* while 3a and 3e are significantly effective against *Salmonella typhi* at most of the six concentrations used. As a consequence it can be concluded that 3d, 3a and 3e synthesized azo dyes containing 2, 4-dinitrophenol moiety, may be used for the development of new antibacterial drugs to cure many disorders caused by the different pathogenic bacterial species.

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REFERENCES

[1] J. Koh and A.J. Greaves, *Dyes Pigments;* **2001**, 50,117
[2] N. Sekar, *Colourage;* **1999**, 46**,** 63
[3] H.A. Dabbagh, A. Teimouri, A. R. Najafi Chermahini, *Dyes Pigments*, **2007**, 73, 239-244.
[4] T. Robinson, G. McMullan, R. Marchant, P. Nigam, Bioresource Technol. **2001**, 77, 247-255.
[5] G. Viscardi, P. Quagliotto, C. Barolo, G. Caputo, G. Digilio, I. Degani, E. Barni, *Dyes Pigments* **2003**, 57, 87-95.
[6] A. Fraleoni-Morgera, C. Della-Casa, P. Costa-Bizzarri, M. Lanzi, A. Missiroli, *Macromolecule*, **2005**, 38 3170-3175.
[7] H. Zolinger, Diazo and Azo Chemistry, *Interscience publishers Inc*. New York, **1961**.
[8] G. Pandey and K.K. Narang . *Bioinorganic Chemistry and Applications* **2005**, Vol. 3, 217-238.
[9] A.V. Patel. *E-journal of chemistry*, **2009**, Vol. 6(4), 1247-1252.
[10] Shridhari A H, Keshavayya H, Hoskeri H J, Ali RAS, *Int Res J Pure Appl Chem* **2011**, 1:119-129.
[11] Avci GA, Ozkinali S, Ozluk A, Avci E, Kocaokutgen H, *J Biol Chem* **2012**, 40:119-126.
[12] Gopalakrishnan S, Nevaditha NT, Mythili CV , *J Chem Pharm Res* **2011**, 3:490-497.
[13] Koshti S.M., Sonar J.P., Sonawane A.E., Pawar Y.A., Nagle P.S., Mahulikar P.P. and More D.H., *Indian J. Chem.*, **2008**, 47B, 329-331.
[14] Pathan R.U. and Borul S.B., *Oriental J. Chem.*, **2008**, 24(3), 1147-1148.
[15] Rathod K.M., *Oriental J. Chem.*, **2010**, 26(3), 1163-1166.
[16] Rathod K.M. and Thakare N.S., *Chem. Sci. Trans.*, **2013**, 2 (1), 25-28.
[17] Rathod K.M., *Asian J. Research Chem.*, (**2011**), 4(5), 734-736.
[18] Pagariya R.F., Rathod K.M. and Thakare N.S., *Asian J. Research Chem.*, **2013**, 6(9), 824-827.
[19] Chatwal G R, Synthetic drugs, Himalaya Publishing house, **1996**, 33*1*.
[20] Koshti S.M., Sonar J.P., Sonawane A.E., Pawar Y.A., Nagle P.S., Mahulikar P.P. and More D.H., *Indian J. Chem.*, 47B, 329-331 (**2008**).
[21] Pathan R.U. and Borul S.B., *Oriental J. Chem.*, 24(3), 1147-1148 (**2008**).
[22] Dahake P.R. and Kamble S.I., *BBRA*, **2014**, 11(3), 1695-1698.
[23] Alzoreky N.S. and Nakahara K., *Int. J. Food Microbial*, **2003**, 80, 223-230