Original Research Article

Antimicrobial, Antioxidant, and Anti-inflammatory evaluation of synthesised azo compounds based on β-naphthol, catechol and quinol nucleus.

Abstract

A series of 10-aryl substituted azo dyes were synthesized by coupling of β-naphthol, catechol, and quinol with different aromatic amines. The synthetic compounds were screened for their in vitro antimicrobial, antioxidant, and anti-inflammatory activities. The characterization of the synthesized compounds was carried out by UV, IR, ^{1}H NMR and Mass spectroscopy. ^{13}C NMR was employed to confirm the structural identity of some of the compounds. After screening for the presence of antimicrobial constituents in the *azo* compounds using HTSPOTi culture method, quinol compounds 7 [(*E*)-2(2nitrophenyl)diazenyl)benzene-1,4-diol] and 10 [(*E*)-2-(3-nitrophenyl)diazinyl)benzene-1,4-diol] were the most active in terms of broad spectrum activity against Gram-positives [*Staphylococcus aureus* (ATCC 25923), *Streptococcus pyrogenes* (clinical strain), *Enterococcus faecalis* (ATCC 29212)],, Gram-negatives [*Salmonella typhi* (clinical strain), *Escherichia coli* (ATCC 251922)] and *Pseudomonas aeruginosa* (ATCC 27853) and the *Candida albicans* at minimum inhibitory concentrations of 15.63-31.25 μg/mL. A quinol derivative, [(*E*)-2-(2nitrophenyl) diazinyl) benzene-1, 4-diol], exhibited the best anti-inflammatory activity in the heat-denatured egg albumin assay. Compounds [ethyl (Z)-4-(3-hydroxynapthalen-1-yl (diazinyl) benzoate] and (E)-2-((3-nitrophenyl) diazenyl) benzene-1,4-diol showed high DPPH free radical scavenging activity during the antioxidant assay.

Keywords: *azo* compounds, antimicrobial, anti-inflammatory, DPPH assay.

1.0 Introduction

Infectious disease cases have been reported to pose a serious global threats emerging every year and continue to resurface as there is a spread of new pathogens coupled with the evolution of already existing pathogens **[1].** However, there have been various challenges during the implementation of policies to help curb the ascendency of infectious diseases. Key amongst them is the development of resistance strains to current chemotherapy against the etiological agents and pathogens of these diseases **[2].** Since the discovery of sulphonamides and penicillin as maiden antibiotics in the 20th century, a wide range of natural and synthetic chemotherapeutic agents have seen the light of the day towards reducing antimicrobial resistance **[3, 4].** Among these chemotherapeutic agents are *azo* dyes which have been employed in various biological activities including antidiabetics, antineoplastics, antiseptics, and antimicrobial agents **[5, 6].** With the enhanced reliability of synthetic tools and screening methods coupled with an urgent quest in combating antimicrobial resistance, the ease of synthesis and biological versatility of *azo* compounds are suitable targets against various pathogens **[7].** Nevertheless, in targeting these microbial infections, essential factors such as inflammation and oxidative stress are implicated, hence understanding the principles underlying these factors involved in the pathogenesis of infections would help in the effective management of various diseases **[8, 9].** Following on this path, most antimicrobial agents are also investigated for their potential antioxidant and anti-inflammatory potentials as part of the process for discovering more potent chemotherapeutic agents.

With the global threat of resistance to current chemotherapy, this research describes the design, synthesis, and evaluation of the antimicrobial, antioxidant, and anti-inflammatory activities of some *azo* compounds in the quest for newer antibiotics. We present herein our findings, which evaluates the antimicrobial, antioxidant, and anti-inflammatory properties of the synthetic *azo* dyes.

2.0 Materials and Methods

2.1 Pathogenic strains

Standard reference strains of pathogens, including; Gram-positive organisms [*Staphylococcus aureus* (ATCC 25923)*,* *Streptococcus pyrogenes* (clinical strain), *Enterococcus faecalis* (ATCC 29212)], Gram-negative organisms [*Salmonella typhi* (clinical strain)*,* *Escherichia coli* (ATCC 251922)] and *Pseudomonas aeruginosa* (ATCC 27853) and fungus *Candida albicans* (ATCC 10231), were used for the antimicrobial study. The test organisms were all obtained from the cell culture laboratory, Department of Pharmacology, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana.

Glycerol stocks of the pathogens were stored at -80 °C in a freezer, were removed, thawed, and cultured either on nutrient and Saboraud agar slants for the bacteria and fungus respectively.

2.2 Culture Media and Reference Antibiotics

Nutrient Agar and Broth, as well as Middlebrook 7H10 Agar and Middlebrook 7H9 Broth, were purchased from Oxoid Limited (Basingstoke, United Kingdom). Ciprofloxacin (Exeter, United Kingdom) and fluconazole were sourced from Sigma Aldrich ™ (Michigan, USA).

2.3 Chemistry

All chemicals used were of synthetic grade and were sourced from Fisher Scientific ™ (United Kingdom) without further purification. The progress of each synthetic reaction was monitored using thin-layer chromatography (TLC), on a precoated silica gel plate (Merck F254) and visualized with UV light (254 nm and 357 nm) or iodine vapour where necessary. The synthesised compounds were purified by recrystallization using suitable solvents. ^{1}H-NMR spectra were obtained with a 500 MHz NMR spectrophotometer (Bruker Biospin NMR Spectrometer, Billerica, U.S/F/NMR/ A 175) using deuterated methanol unless otherwise stated as the solvent and the chemical shifts were reported in δ ppm. The samples were run neat to obtain their infra-red (IR) spectra, in the range 400 – 4000 cm^{-1} on a Bruker FTIR spectrophotometer (Bruker FTIR 94133). The Ultraviolet-Visible (UV-Vis) spectra were measured on a UV-Vis spectrophotometer (Jenway, U.K/7315), at 200 – 800 nm with methanol as a blank and used to determine the λ max of the synthesised compounds. The melting points of the synthesised compounds were determined by the open capillary method on a Stuart melting point apparatus (England) and are uncorrected.

2.4 Synthesis, and Characterization of Azo Compounds

This study included the design and synthesis of analogues of β-naphthol, quinol and catechol *azo* compounds, bearing benzene with different substituents (-NO_2, -COOH, -$COCH_3$) following protocols from **[10]**. The primary aromatic amines (1g) were reacted with $NaNO_2$ (1 M, 20 mL) in the presence of HCl (2 M, 20 mL, 36 % w/v) at 0 – 5 ^{o}C in a salt- ice bath to produce diazonium salts. The clear diazonium solutions were confirmed by the presence of an instantaneous blue colour on testing with starch iodide solution. The diazotised compounds were then coupled with β-naphthol, catechol, and quinol in the presence of NaOH (2.5 M, 200 mL, 98 %) by in an ice bath while stirring to produce the coupled product. The precipitate of the coupled products was filtered by suction and dried and purified by recrystallization from hot ethanol to give coloured solids **1-10,** with yields of 21-98 % w/w as shown in (**Figure 1**).

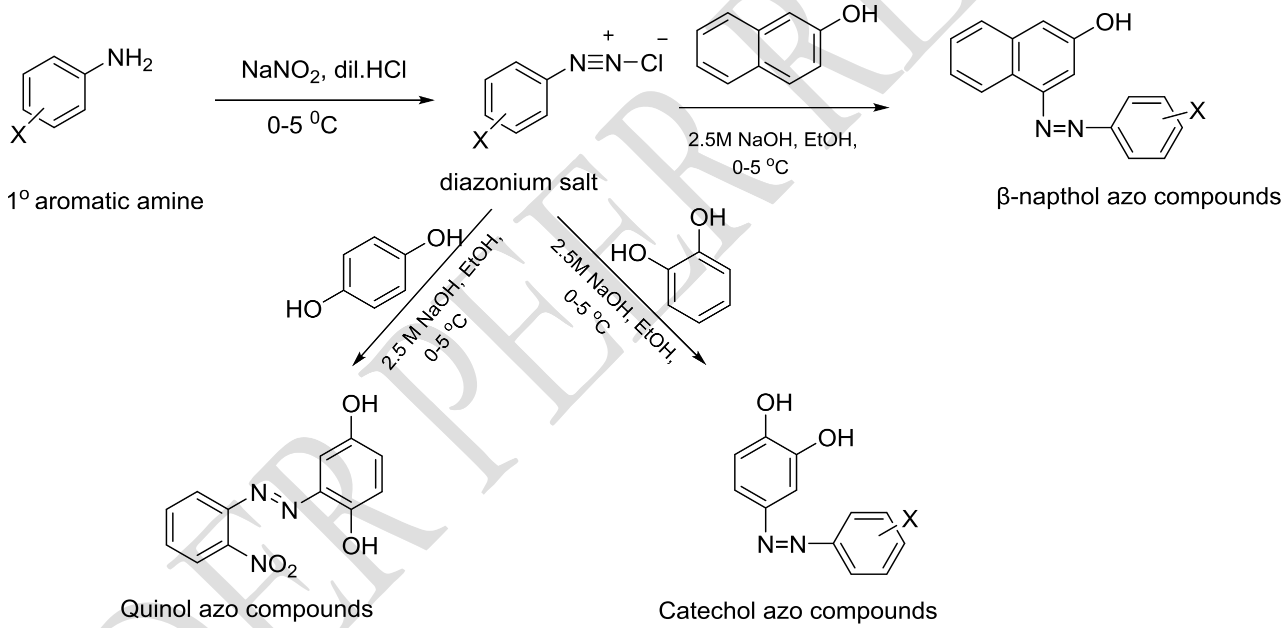


Figure 1: General scheme for the synthesis of the azo compounds; β-naphthol: (X= *p*-COOH), (X= *p*-$COOC_2H_5$), (X=*p*-Cl), (X= *p*-SO_2NH_2), (X=*o*-NO_2) (X=*m*-NO_2), Quinol: (X= *m*-NO_2), (X= *o*-NO_2); Catechol: (X= *m*-NO_2), (X= *o*-NO_2). *o*, *m,* and *p* represent ortho, meta, and para substitution, respectively.

2.5 Antimicrobial Activity of Synthesised Azo Compounds

Evaluation of the synthesized compounds for possible antimicrobial activity was *via* the High throughput spot culture growth inhibition test (HT-SPOTi) well defined by **[11]** Danquah *et al*., 2016) which employs a sterile 96-well microplate for the assay.

2.5.2 Susceptibility of Test Organisms to synthesised compounds

Different concentrations of the *azo* compounds were tested against a panel of [*Staphylococcus aureus* (ATCC 25923), *Streptococcus pyrogenes* (clinical strain), *Enterococcus faecalis* (ATCC 29212)], Gramnegative organisms [*Salmonella typhi* (clinical strain), *Escherichia coli* (ATCC 251922)] and *Pseudomonas aeruginosa* (ATCC 27853) and fungus *Candida albicans* (ATCC 10231) to determine their MICs using the high-throughput spot culture growth inhibition assay (HT-SPOTi). In the HT-

SPOTi assay, molten agar maintained at 55-60 °C was dispensed into 96-well plates to which 2 μL of serially diluted *azo* compounds have been added from a stock of 50 mg/mL. The bacterial suspension (2 μL; 1 × 10^6 cfu/mL) was added to each plate, sealed, and incubated for 18 – 24 h. The lowest concentration at which bacterial growth was completely inhibited by the compound was observed visually, and the MIC was recorded.

2.6 Anti-inflammatory activity

The protocol of **[12]** was followed for the anti-inflammatory assay. Prior to the assay, test tubes were obtained, cleaned, and dried as they are the vessels in which the reactions were carried out. The reaction mixture (5 mL) consisted of 2.8 mL of phosphate buffer of pH 6.8, 0.2 mL of egg albumin (from fresh hen's egg), and 2 mL of varying concentrations of the synthesized compounds such that the final concentrations were 10 mg/mL, 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL and 0.625 mg/mL. Similar volumes of egg albumin, phosphate buffer, distilled water, and dimethylsulphoxide (DMSO) served as negative control. These mixtures were incubated at 37 °C in an incubator for 30 min and then heated at 75 °C for 10 min. Their absorbances were then measured at 660 nm after cooling. The reference drug, diclofenac was treated similarly to obtain five different concentrations for determination of absorbance. The experiment was carried out in triplicate and the percentage inhibition of protein denaturation was calculated by using the following formula:

$$\%\ inhibition = (Abs_{control} - Abs_{treated}) / Abs_{control} \times 100 \ldots\ldots Equation\ 3.1$$

Where Abs $_{treated}$ = Absorbance of test sample, Abs $_{control}$ = Absorbance of control. The drug concentration for 50 % inhibition (IC_{50}) was determined by plotting the percentage inhibition with respect to control against treatment concentration.

2.7 Antioxidant activity

The antioxidant activity of the synthesized compounds was determined by DPPH method **[13]**. 1 mg/1.0 mL solutions of the various synthesized *azo compounds* were prepared. 50 μL solution of each of the compounds synthesized was added to 2 mL of freshly prepared 0.2 mM DPPH solution in methanol. The resulting solutions obtained from the above were incubated for 20 min at 37 °C. Their individual absorbances were taken at 517 nm using the double beam spectrophotometer. Ascorbic acid (1 mM) was employed as a positive control. The percentage scavenging activity of the synthesized *azo compounds* was calculated by using the following formula.

% inhibition= $\frac{(abs.of\ the\ control-abs\ of\ the\ test\ sample)}{abs.of\ the\ control}\times \mathbf{100}$ ...**Equation 3.2** (Bag *et al*., 2015)

2.8 Statistical Analysis

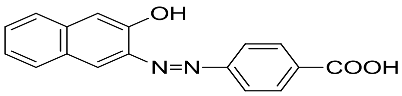
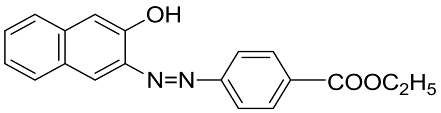
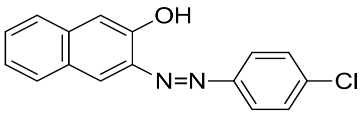
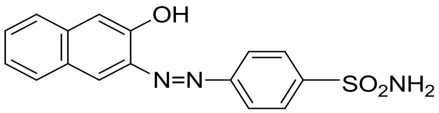
The data from the study were analyzed using GraphPad Prism (Version 8.01, GraphPad Software Inc., USA). The data were described using descriptive statistics and tested inferentially using One-Way analysis of variance (ANOVA) with the Neuman-Keuls post-test. The levels of significance were set at $p < 0.05$ in all scenarios.

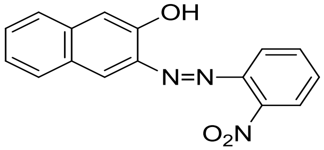
3.0 Results and discussion

3.1 Characterization of synthesised Azo Compounds

The synthesis of the library of azo dyes **(1-10)** (**Table 1; Figure 1**) was performed according to methods in the literature. The scaffold design for the compounds is illustrated in **Figure 2.** The compounds with Cl, NO_2, COCH3, and COOH substituents including naphthol, quinol, and catechol nuclei were chosen because of their potential antimicrobial activities in studies as reported by works from [32] and [33]. The compounds were obtained in good yields and high purity confirmed by Thin Layer Chromatography, melting point, and spectroscopic methods. The compounds 1-10, known and reported in the literature were confirmed based on IR, UV-Vis, and ^1H NMR spectroscopic data [34]. For instance, the IR spectrum of **compound 7** showed vibrational bands for OH and N=N at 3451 and 1397 cm^{-1} respectively. The UV-Vis absorption spectrum of 365 nm confirmed the presence of extended chromophores after the molecular hybridization coupling process. The ^1H NMR spectrum showed an envelope of seven aromatic protons resonating downfield. The sharp uncorrected melting points confirmed the purity of the compounds. The visible colours of the compounds were due to the presence of extended conjugation, which increases the wavelength of absorption. Moreover, the different colours suggested the presence of varying substituents and their positions on the chromophore system (**Table 1**). This was confirmed by their UV-Vis absorption spectra in the supporting information.

Table 1: Physical properties of the azo compounds

COMPOUNDS	*CODES*	*MOLECULAR FORMULA*	*MOLECULAR WEIGHT (G/MOL)*
	**36**	$C_{17}H_{12}N_2O_3$	292.29
	**38**	$C_{19}H_{16}N_2O_3$	320.34
	**40**	$C_{16}H_{11}ClN_2O$	282.72
	**42**	$C_{16}H_{13}N_3O_3S$	320.34

	**44**	$C_{16}H_{11}N_3O_3$	293.28

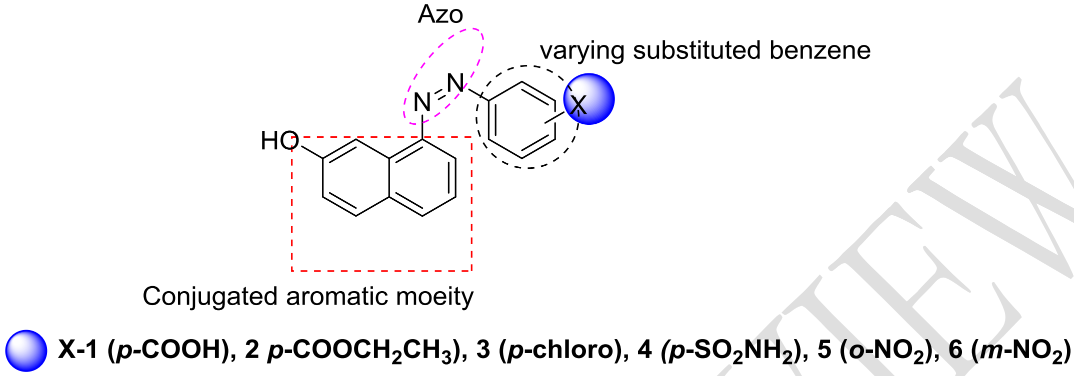


Figure 2: Sample scaffold design for naphthol *azo* compounds **1-6**

4-((3-hydroxynaphthalen-1-yl)diazenyl)benzoic acid [1]

The reaction of *p*-aminobenzoic acid (2.130 g, 7.30 mmol, 2.09 eq.), and $NaNO_2$ (1 M, 20 mL, 20 mmol, 5.71 eq.) in HCl (36 % w/w, 50 mL, 50 mmol, 14.23 eq.) and coupling with β-naphthol (1eq. 0.45 mmol) produced a red solid, yield 0.987 g (95 %w/w) and the purity monitored by thin layer chromatography; $R_f = 0.72$ (EtOAc: MeOH, 5:5 v/v); mp: 293-295 °C. $C_{17}H_{12}N_2O_3$ (292.4 29 g/mol). UV-Vis (λmax/ nm): 488; IR ν_{max}/cm^{-1} (% T): -COOH (3385, 87 %), -N=N (1427, 70 %), aromatic C=C- (1601 (72 %), 1498 (66 %), 833 (64 %); ^{1}H NMR (500 MHz, $CDCl_3$, rt); δ_H [ppm] = 6.71 (1H, d, *J* = 9.0 Hz, C(8) H_{Ar}), 7.38 (1H, s, C(1) H_{Ar}), 7.52 (2H, d, *J* = 6.5 Hz, C(1', 3') H_{Ar}), 7.66 (3H, s, C(5, 6, 7) H_{Ar}), 8.10 (2H, d, *J* = 6.5 Hz, C(2', 4') H_{Ar}), 8.43 (1H, s, C(4) H_{Ar}).

Ethyl 4-((3-hydroxynaphthalen-1-yl) diazenyl) benzoate [2]

The reaction of ethyl *p*-aminobenzoate (1.008 g, 6.10 mmol, 1.74 eq.) and $NaNO_2$ (1 M, 20 mL, 20 mmol, 5.71 eq.) in HCl (36 % w/w, 50 mL, 50 mmol, 14.29 eq.) and coupling with β-naphthol (1eq. 0.45 mmol) produced an orange solid, yielding 0.5680 g (51 %w/w) and the purity monitored by thin layer chromatography; $R_f = 0.64$ (EtOAc: Petroleum ether, 0.5:9.5 v/v); mp: 158-160 °C. $C_{19}H_{16}N_2O_3$ (320.34 g/mol). UV-Vis (λmax/ nm): 495; IR ν_{max}/cm^{-1} (% T): -N=N (1397 (88 %), aromatic C=C- (1619 (89 %), 1450 (87 %), 761 (79 %), ester C=O (1711 (84 %), ^{1}H NMR (500 MHz, $CDCl_3$, rt); δ_H [ppm] = 1.41 (3H, m, C(2'') H_3), 4.39 (2H, m, C(1'') H_2), 6.74 (1H, m, C(7) H_{Ar}), 7.41 (1H, m, C(1) H_{Ar}), 7.55 (2H, m, C(1', 3') H_{Ar}), 7.66 (1H, m, C(5) H_{Ar}), 7.67 (1H, m, C(6) H_{Ar}), 7.69 (1H, m, C(8) H_{Ar}), 8.12 (2H, m, C(2', 4') H_{Ar}), 8.45 (1H, m, C(4) H_{Ar}); ^{13}C NMR (500 MHz, $CDCl_3$, rt) δ_C [ppm] = 14.46 C(2''), 61.16 C(1''), 116.96 (Ar-C), 122.23 (Ar-C), 126.17 C(7), 126.84 C(6), 129.01 C(8), 129.53 C(5), 131.38 C(1), 133.48 C(3), 142.41 C(1', 3'), 146.77 C(2', 4'), 178.05.

***4-((4-chlorophenyl)diazenyl)naphthalen-2-ol* [3]**

The reaction of *p*-chloroaniline (0.9950 g, 7.8 mmol, 2.23 eq.) and $NaNO_2$ (1 M, 20 mL, 20 mmol, 5.71 eq.) in HCl (36 % w/w, 50 mL, 50 mmol, 14.29 eq.) and and coupling with β-naphthol (1eq. 0.45 mmol produced a red solid, yield 0.854 g (98 %w/w) and the purity monitored by thin layer chromatography; R_f = 0.48 (EtOAc: Petroleum ether, 0.2:9.8 v/v) mp: 171-174 °C. $C_{16}H_{11}ClN_2O$ (282.72 g/mol). UV-Vis (λmax/ nm): 486; IR ν $_{max}$/cm^{-1} (% T): -N=N (1387 (75 %), aromatic C=C- (1600 (84 %), 1559 (79 %), 780 (97 %), C-Cl (677 (74 %); ^{1}H NMR (500 MHz, $CDCl_3$, rt); δ_H (ppm) 6.87 (1H, m, C(7) H_{Ar}), 7.39 (1H, m, C(8) H_{Ar}), 7.43 (2H, m, C(1', 3') H_{Ar}), 7.55 (1H, t, *J* = 1.2 Hz C(6) H_{Ar}), 7.60 (1H, m, C(5) H_{Ar}), 7.66 (2H, m, C(2', 4') H_{Ar}), 7.72 (1H, d, *J* = 9.4 Hz, C(1) H_{Ar}), 8.53 (1H, m, C(4) H_{Ar}); ^{13}C NMR (500 MHz, $CDCl_3$, rt); δ_C [ppm]= 119.00 (Ar-C), 120.89 (Ar-C), 127.29 C(7), 128.06 C(6), 128.87 C(8), 129.32 C(5), 132.08 C(1), 132.49 C(3), 139.25 C(1', 3'), 142.93 C(2', 4').

***4-((3-hydroxynaphthalen-1-yl) diazenyl) benzenesulfonamide* [4]**

The reaction of sulphanilamide (0.9990 g, 5.80 mmol, 1.66 eq.) and $NaNO_2$ (1 M, 20 mL, 20 mmol, 5.71 eq.) in HCl (36 % w/w, 50 mL, 50 mmol, 14.29 eq.) and coupling with and coupling with β-naphthol (1eq. 0.45 mmol) produced an orange solid, yielded 1.606 g (72 %w/w) and the purity monitored by thin layer chromatography; R_f = 0.7 (EtOAc: Petroleum ether, 5:5 v/v) mp: 171-174 °C. $C_{16}H_{13}N_3O_3S$ (327.36 g/mol). mp:187-190 °C; UV-Vis (λmax/ nm): 480; IR ν $_{max}$/cm^{-1} (% T): N-H (3317 (97 %), N=N (1339 (95 %), aromatic C=C- (1618, (95 %), 1519 (91 %), 825 (89 %); ^{1}H NMR (500 MHz, $CDCl_3$, rt); δ_H (ppm) 6.75 (1H, m, C(7) H_{Ar}), 7.40 (1H, m, C(6) H_{Ar}), 7.61 (1H, d, *J* = 7.5 Hz, C(1) H_{Ar}), 7.74 (4H, m, C(1', 2', 3', 4') H_{Ar}), 7.95 (2H, d, *J* = 8.6 Hz, C(5, 8) H_{Ar}), 8.46 (1H, d, *J* = 8.1 Hz, C(4) H_{Ar}

***4-((2-nitrophenyl) diazenyl) naphthalen-2-ol* [5].**

The reaction of 2-nitroaniline (0.9991 g, 7.24 mmol, 1.61 eq.) and $NaNO_2$ (1 M, 20 mL, 20 mmol, 4.44 eq.) in HCl (36 % w/w, 50 mL, 50 mmol, 11.11 eq.) and coupling with and coupling with β-naphthol (1eq. 0.45 mmol) produced a bright brown solid, yielding 1.4218 g (67 %) and the purity monitored by thin layer chromatography; R_f = 0.82 (EtOAc: Petroleum ether, 5:5 v/v) mp: 110-153 °C. $C_{12}H_9N_3O_4$ (259.22 g/mol). UV-Vis (λmax/ nm): 405; IR ν $_{max}$/cm^{-1} (% T): -N=N (1352.4, 53.99 %), -NO_2 (1526.93, 50.47 %), aromatic C=C- (1602.65, 89.17 %), (1585.84, 88.95 %), (757.01, 83.82 %), aromatic –OH stretch (3401.81, 93.51 %; ^{1}H NMR (500 MHz, MeOD, rt); δ_H (ppm) = 7.90 (1H, d, *J* = 4.0 Hz, C(2') H_{Ar}), 7.91 (2H, d, *J* = 2.6 Hz, C(1', 5) H_{Ar}), 7.93 (1H, d, *J* = 4.2 Hz, C(3') H_{Ar}), 8.07 (1H, d, *J* = 4.2 Hz C(6) H_{Ar}), 8.09 (1H, d, *J* = 2.6 Hz, C(3) H_{Ar}), 8.10 (1H, d, *J* = 4.1 Hz, C(4') H_{Ar}).

(E)-4-[(2-nitrophenyl) diazenyl] benzene-1,2-diol [6]

The reaction of 2-nitroaniline (0.9991 g, 7.24 mmol, 1.61 eq.) and $NaNO_2$ (1 M, 20 mL, 20 mmol, 4.44 eq.) in HCl (36 % w/w, 50 mL, 50 mmol, 11.11 eq.) and coupling with catechol (1 eq., 4.50 mmol) produced a bright brown solid, yield 1.4218 g (67 %) and the purity monitored by thin layer chromatography; R_f = 0.82, (EtOAc: Petroleum ether, 5:5 v/v) mp: 110-153 °C. $C_{12}H_9N_3O_4$ (259.22 g/mol). UV-Vis (λmax/ nm): 405; IR ν_{max}/cm^{-1} (% T): -N=N (1352.4, 53.99 %), -NO_2 (1526.93, 50.47 %), aromatic C=C- (1602.65, 89.17 %), (1585.84, 88.95 %), (757.01, 83.82 %), aromatic –OH stretch (3401.81, 93.51 %); The ^{1}H NMR spectrum showed only these signals: ^{1}H NMR (500 MHz, MeOD, rt); δ_H (ppm) 7.90 (1H, d, *J* = 4.0 Hz, C(2') H_{Ar}), 7.91 (2H, d, *J* = 2.6 Hz, C(1', 5) H_{Ar}), 7.93 (1H, d, *J* = 4.2 Hz, C(3') H_{Ar}), 8.07 (1H, d, *J* = 4.2 Hz C(6) H_{Ar}), 8.09 (1H, d, *J* = 2.6 Hz, C(3) H_{Ar}), 8.10 (1H, d, *J* = 4.1 Hz, C(4') H_{Ar}).

(E)-2-[(2-nitrophenyl)diazenyl]benzene-1,4-diol [7]

The reaction of 2-nitroaniline (0.9991 g, 7.24 mmol, 1.61 eq.) and $NaNO_2$ (1 M, 20 mL, 20 mmol, 4.44 eq.) in HCl (36 % w/w, 50 mL, 50 mmol, 11.11 eq.) and quinol ((1 eq., 4.50 mmol) produced a bright brown solid, yield 0.5305 g (25 %) and the purity monitored by thin layer chromatography; $R_{f.}$ = 0.80, (EtOAc: Petroleum ether, 5:5 v/v) mp: 84-86 °C. $C_{12}H_9N_3O_4$ (259.22 g/mol). UV-Vis (λmax/ nm): 450; IR ν_{max}/cm^{-1} (% T): -N=N (1351.60, 83.75 %), -NO_2 (1525.85, 80.95 %), aromatic C=C- (1451, 93.54 %), (789, 87.54%), aromatic –OH stretch (3401.81, 97.84; ^{1}H NMR (500 MHz, MeOD, rt); δ_H (ppm)7.90 (1H, d, *J* = 4.0 Hz, C(5) H_{Ar}), 7.91 (2H, d, *J* = 2.6 Hz, C(2', 3') H_{Ar}), 7.92 (1H, d, *J* = 4.1 Hz, C(4) H_{Ar}), 8.07 (1H, s, C(6') H_{Ar}), 8.08 (1H, d, *J* = 2.5 Hz, C(6) H_{Ar}), 8.10 (1H, d, *J* = 3.9 Hz, C(3) H_{Ar}).

(E)-3-[(3-nitrophenyl)diazenyl]naphthalen-2-ol [8]

The reaction of 3-nitroaniline (0.9991 g, 7.24 mmol, 2.07 eq.) and $NaNO_2$ (1 M, 20 mL, 20 mmol, 5.71 eq.) in HCl (36 % w/w, 50 mL, 50 mmol, 14.29 eq.) produced a bright orange solid, yield 1.5915 g (72 %) and the purity monitored by thin layer chromatography; R_f: = 0.68, (EtOAc: Petroleum ether, 1:9 v/v) mp: 168-170 °C. $C_{16}H_{11}N_3O_3$ (293.28 g/mol). UV-Vis (λmax/ nm): 395; IR ν_{max}/cm^{-1} (% T): -N=N (1398.81, 77.5 %), NO_2 (1525.92, 64.34 %), aromatic C=C- (1498.34, 61.96 %), (839.53, 69.64 %); ^{1}H NMR (500 MHz, MeOD, rt); δ_H (ppm) 6.83 (1H, d, *J* = 9.5 Hz, C(1) H_{Ar}), 7.68 (1H, s, C(4) H_{Ar}), 7.76 (2H, t, *J* = 8.1 Hz, C(6, 7) H_{Ar}), 7.87 (1H, s, C(5) H_{Ar}), 7.89 (1H, s, C(8) H_{Ar}), 8.13 (3H, m, C(1', 2', 3') H_{Ar}), 8.55 (1H, d, *J* = 8.1 Hz, C(5') H_{Ar}).

4-[(3-nitrophenyl) diazenyl] benzene-1,2-diol *[9]*

The reaction of 3-nitroaniline (0.9991 g, 7.24 mmol, 1.61 eq.) and $NaNO_2$ (1 M, 20 mL, 20 mmol, 4.44 eq.) in HCl (36 % w/w, 50 mL, 50 mmol, 11.11 eq) produced a bright brown solid, yield 1.2250 g (58 %) and the purity monitored by thin layer chromatography; R_f: = 0.81, (EtOAc: Petroleum ether, 5:5 v/v) mp: 138-140 °C. $C_{12}H_9N_3O_4$ (259.22 g/mol). UV-Vis (λmax/ nm): 390; IR ν_{max}/cm^{-1} (% T): -N=N (1344.95, 58.31 %), -NO_2 (1524.6, 64.31 %), aromatic –OH stretch (3401.81, 85.21 %); ^{1}H NMR (500 MHz, $CDCl_3$, rt); δ_H (ppm) 7.41 (1H, d, *J* = 5.3 Hz, C(3) H_{Ar}), 7.81 (3H, m, C(1', 2', 3') H_{Ar}), 8.52 (1H, d, *J* = 5.3 Hz, C(5) H_{Ar}), 8.54 (1H, d, *J* = 5.3 Hz, C(6) H_{Ar}), 8.90 (1H, t, *J* = 2.2 Hz, C(5') $H_{Ar.}$

(*E*)-2-[(3-nitrophenyl)diazenyl]benzene-1,4-diol [10]

The reaction of 3-nitroaniline (0.9991 g, 7.24 mmol, 1.61 eq.) and $NaNO_2$ (1 M, 20 mL, 20 mmol, 4.44 eq.) in HCl (36 % w/w, 50 mL, 50 mmol, 11.11 eq.) and coupling with quinol (1eq., 0,45 mmol) produced a bright brown solid, yield 0.4456 g (21 %) and the purity monitored by thin layer chromatography; R_f: = 0.50, (EtOAc: Chloroform, 5:5 v/v) mp: 95-96 °C. $C_{12}H_9N_3O_4$ (259.22 g/mol). UV-Vis (λmax/ nm): 365; IR ν_{max}/cm^{-1} (% T): -N=N (1397.9, 87.5 %), -NO_2 (1521, 63.44 %), aromatic C=C- (1613.81, 87.24 %), (1447.04, 86.53 %), (774.34, 86.59 %), aromatic –OH stretch (3451.81, 94.35 %); ^{1}H NMR (500 MHz, $CDCl_3$, rt); δ_H (ppm) 6.81 (1H, m, C(6') H_{Ar}), 7.63 (1H, m, C(6) H_{Ar}), 7.93 (2H, m, C(2', 3') H_{Ar}), 8.64 (1H, d, *J* = 2.0 Hz, C(4) H_{Ar}), 8.65 (1H, d, *J* = 2.2 Hz, C(5) H_{Ar}), 9.02 (1H, t, *J* = 2.2 Hz, C(2) H_{Ar}).

Table 2: *Structures and physical data of the synthesized azo compounds*

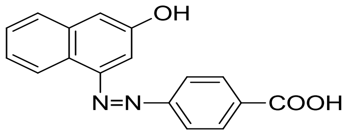
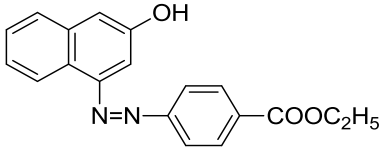
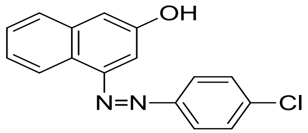
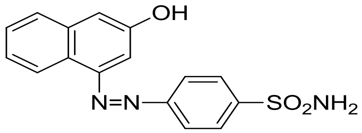
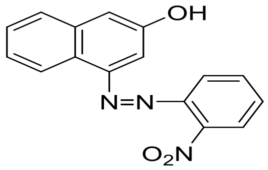
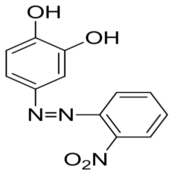
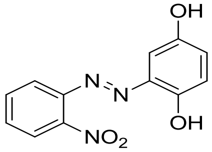
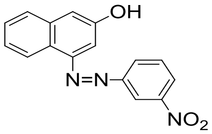
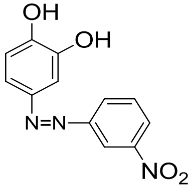
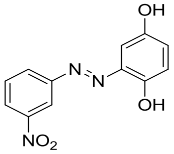
COMPOUNDS	*CODES*	*MOLECULAR FORMULA*	*MOLECULAR WEIGHT (G/MOL)*	*YIELD (%)*	*MELTING POINT (^{O}C)*	*R_F VALUE*
	1	$C_{17}H_{12}N_2O_3$	292.29	95.17	293-295	0.72
	2	$C_{19}H_{16}N_2O_3$	320.34	51.13	158-160	0.64
	3	$C_{16}H_{11}ClN_2O$	282.72	98.05	171-174	0.48
	4	$C_{19}H_{16}N_2O_3$	320.34	72.3	187-190	0.7
	5	$C_{16}H_{11}N_3O_3$	293.28	85	153-155	0.64

TABLE 2 Cont

	6	$C_{12}H_9N_3O_4$	259.22	67	110-113	0.82
	7	$C_{12}H_9N_3O_4$	259.22	25	84-86	0.80
	8	$C_{16}H_{11}N_3O_3$	293.28	72	168-170	0.68
	9	$C_{12}H_9N_3O_4$	259.22	58	138-140	0.81
	10	$C_{12}H_9N_3O_4$	259.22	21	95-96	0.50

3.3 Antimicrobial Activity of Synthesised Azo Compounds

Table 3: Minimum inhibitory concentrations (µg/mL) against the micro-organisms

Minimum inhibitory concentrations (µg/mL) against the micro-organisms

	Gram-negative bacteria			***Gram-positive bacteria***			***Fungus***
COMPOUNDS	***Salmonella. typhi***	***Escherichia coli***	***Pseudomonas aeruginosa***	***Staphylococcus aureus***	***Streptococcus pyrogenes***	***Enterococcus faecalis***	***Candida albicans***
1	62.50	>500	>500	125	250	500	>500
2	>500	62.5	>500	>500	62.5	> 500	500
3	>500	>500	>500	>500	>500	> 500	>500
4	>500	31.25	>500	250	500	> 500	>500
5	250	62.5	250	15.63	31.25	15.63	15.63
6	125	31.25	125	125	125	31.25	125
7	15.63	15.63	31.25	31.25	125	15.63	62.5
8	62.50	15.63	125	250	15.63	125	250
9	31.25	125	125	62.5	31.25	62.5	125
10	15.63	125	15.63	62.5	31.25	31.25	62.5
Ciprofloxacin	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	7.8	125	nd
Fluconazole	nd	nd	nd	nd	nd	nd	≤ 0.5

Key: nd (not determined for the particular organism)

Synthetic azo dyes have been shown to possess varying degree of antimicrobial activity against several strains of microbes **[15]**. Following on this trajectory, the *in vitro* screening of ten azo compounds [1-10] by employing HTSPOTi culture sensitivity method revealed the presence of antimicrobial constituents against *Salmonella typhi* (clinical strain), *Escherichia coli* (ATCC 251922), *Pseudomonas aeruginosa* (ATCC 27853) [Gram-negatives] *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyrogenes* (clinical strain), *Enterococcus faecalis* (ATCC 29212) [Gram-positive] and *Candida albicans* (ATCC 10231) [fungi].

Pseudomonas aeruginosa was the most resistant especially to naphtholic compounds [1-4] [MIC > 500 µg/mL]. The associated resistance could be attributed to the presence of efflux pumps and porins which are present in Gramnegative bacteria to prevent the entry of harmful chemicals [15, 16]. The presence of an external envelope around the cell and genetic mutations [17] could also contribute to increased resistance of *Pseudomonas aeruginosa* to the naphtholic *azo* compounds. These general features of Gram-negatives make their infections difficult to treat compared to Gram-positive related diseases [18]. It is however interesting to note that despite the low activity of the naphtholic series of compounds, a nitro substitution at the *ortho* position to the naphthol group improved the susceptibility of *Pseudomonas aeruginosa*. The presence of a nitro group at *meta* position on the naphthol improved the antipseudomonal activity by 50 %. The antimicrobial activity of nitro groups has been shown to be due to the production of nitrous and superoxide free radicals from metabolic activation *in vivo* which interferes with the redox cycle and cause microbial paralysis. Metabolic activation of the nitro compound by *Pseudomonas* could have produced these free radicals which contributed to the antibacterial activity [19]. This suggests the essential role of the nitro group and its substitution pattern in inhibiting this resistant organism [20]. Another evidence according to [21], the azo dye 1-(2-carboxyphenylazo)-2-naphthol) exhibited impressive antipseudomonal activity as a result of the substitution pattern. However, compounds 5-10 showed improved activity against resistant *Pseudomonas aeruginosa* with the most active set being the catechol and quinol series with compound 10 (MIC of 15.63 µg/mL) being the most active emphasizing the crucial role of the nitro group. This was also evident in its impressive activity against *Salmonella typhi* at 15.63 µg/mL of its quinol partner, with nitro group at *ortho* group to the quinol. This characteristic of catechol and quinol suggests that the presence of two hydroxyl groups on the benzene ring improved hydrogen bonding which could have been responsible for the improved ligand (catechol quinol) binding at the receptor site of the Gram-negative bacteria.

Of the Gram-negatives, the clinical strain *Salmonella typhi* showed more resistance after *Pseudomonas aeruginosa* with compound 2-4 exhibiting the least activity at > 500 µg/mL. Compounds 1 and 8 which are naphtholic compounds and possess carboxylic acid and *m*-nitroaniline moieties respectively had an improved activity at 62.5 µg/mL. However, the catechol and quinol derivatives had better activity than their naphthol counterparts with quinol compounds 7 and 10 exhibiting the best activity at 15.63 µg/mL. This suggests and emphasise that the presence of two hydroxyl groups for improved ligand-receptor binding *via* hydrogen bonding is very significant for activity against Gram-negatives including the clinical strain *Salmonella typhi.*

Amongst the naphtholic series, compounds 1 and 3 which possess carboxylic acid and *p*-chloro groups had the least activity against *Escherichia coli*. The presence of the ester, ethyl benzoate group in place of the carboxylic acid group greatly improved activity at 62.5 µg/mL similar to *ortho*-nitro group in compound 5 which also had an MIC of 62.5 µg/mL. The naphtholic compound 4 with the sulphonamide moiety was more active than carboxylic acid, ethylbenzoate and the *para* chloro groups. This shows the importance of $-SO_2NH_2$ group for activity against *Escherichia coli*. However, the presence of *meta* nitro group in the naphthol series gave the compound 8 an impressive activity at 15.63 µg/mL against the same organism. Similar activity at 15.63 µg/mL was reported for *the ortho* nitro group in the quinol series. This signifies that the ortho nitro group is essential for activity against *Escherichia coli.* The presence of an electron withdrawing nitro group at the ortho position creates positive centres on the aromatic ring for binding to receptor sites of the organism [22]. Nevertheless, there could be the possibility of nitrous free radicals on metabolic activation by the organism which could have caused paralysis. Hence, substitution pattern and the presence of electron withdrawing nitro group is therefore essential for activity against *Escherichia coli.*

Considering the Gram-positive organisms which possess double-layered peptidoglycan cell walls, *Enterococcus faecalis* (ATCC 29212) was the least susceptible to naphtholic compounds 1-4 at an MIC of 500 to > 500 µg/mL. However, an *ortho* nitro substitution in naphtholic compound 5 improved the activity greatly against this organism similar to compound 7 (quinol derivative) which also possess an *ortho* nitro group. Compounds 5 and 7 were the most active against *Enterococcus faecalis*, followed by compounds 6 (catechol-*ortho* nitro derivative) and 10 (quinol-

meta nitro derivative). This suggests that the presence of a nitro group at the *ortho* and *meta* positions in naphtholic, catechol and quinol *azo* compounds is essential for activity against Gram-positive *Enterococcus faecalis.*

However, the antibacterial activity of the naphtholic *azo* compounds was better against *Staphylococcus aureus* and *Streptococcus pyrogenes*. For instance, compounds 2, 5 and 8 which possess the naphtholic group had improved activity against *Streptococcus pyrogenes* at 62.5 µg/mL, 31.25 µg/mL and 15.63 µg/mL respectively with compound 5 having the best activity against *Staphylococcus aureus* at 15.63 µg/mL. The presence of lipophilic ethyl benzoate group and naphthalene could have improved the permeability of compound 2 across the phospholipid-peptidoglycan cell wall leading to improved antibacterial activity. Moreover, the presence of nitro groups creates positive centres on the aromatic ring, hence improves binding to nucleophiles at the receptor sites of the microorganism. Coupled with the hydrophobic nature of the naphthalene group, ability of compounds 8 to cross the lipophilic barriers was easier, especially for *Staphylococcus aureus* and *Enterococcus faecalis*. However, *ortho*-nitro naphthol group [compound 5] had better activity against *Staphylococcus aureus* and *Enterococcus faecalis* than *meta*-nitro substituted compound 8 emphasising the importance of substitution pattern in improving antibacterial activity. Compound 7 (quinol-2-nitroaniline) also had impressive activity against *E. faecalis* at 15.63 µg/mL. Compound 5 and 7 had better activity than ciprofloxacin (positive control) against *E. faecalis.*

Antifungal activity against *Candida albicans* demonstrated that amongst the naphtholic compounds, the *ortho* nitro group exhibited the best activity at 15.63 µg/mL. This was followed by quinol derivatives [compounds 7 and 10] which possess *ortho* nitro and *meta* nitro groups, respectively. The nitro groups at *ortho* positions coupled with the naphthol and quinol moieties are therefore essential for activity against *Candida albicans.* Catechol compounds [6 and 9] followed in terms of antifungal activity at 125 µg/mL. Groups like carboxylic acid in [compound 1], ethyl benzoate [compound 2], *p*-chloro [compound 3] and sulphonamide [compound 4] reduced drastically the antifungal activity.

It was also observed that compound 3 which is the *p-chloro* (2-naphthol) derivative, had the least activity against all the micro-organisms hence would require optimization in future.

It can be deduced that the antimicrobial activity of the azo compounds may be due to the various substitution patterns on the aromatic ring. Although the exact mechanism of these *azo* compounds have not been investigated, their mode of action may involve various targets such as cell wall and cell membrane interference [23, 24].

The search for synthetic antimicrobial agents has received much attention and considerable input by stakeholders in the field of drug discovery would help achieve this goal [25]. With the current trend in the drug discovery pipeline, these *azo* compounds would provide another source of inspiration in the wake of antimicrobial resistance.

3.4 Anti-inflammatory activity

In this study, egg albumin denaturing bioassay was selected for *in vitro* assessment of anti-inflammatory activities of the synthesized naphtholic catechol and quinol *azo* compounds (Fig. 3).

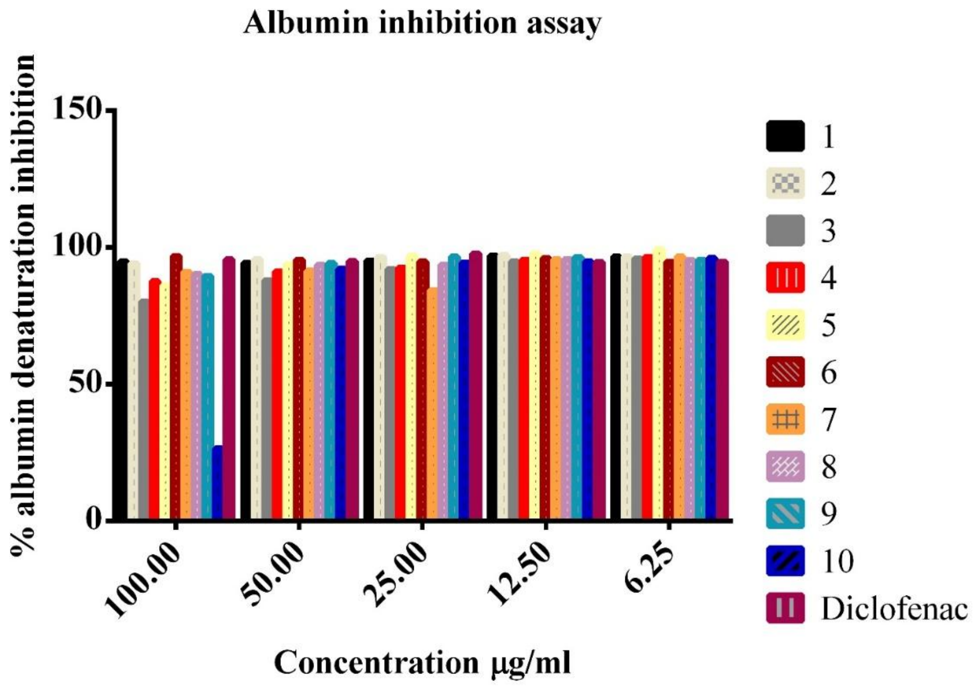


Figure 3: Albumin denaturing inhibition (anti-inflammatory activity) by the synthesized *azo* compounds [1-10]. diclofenac [reference drug].

Denaturing of proteins in tissues is one of the well-known causes of inflammatory diseases. Agents that can prevent the inflammatory cascade *via* protein denaturing would be vital in anti-inflammatory drug discovery. The anti-inflammatory activity of the *azo* compounds and their IC_{50} values were compared with the standard drug diclofenac. From the IC_{50} values, it became evident that the *azo* compounds being effective at lower concentrations inhibited heat-induced protein (albumin) denaturation better than diclofenac. Compound **10**, a quinol *azo* compound with *meta* nitro group had the least anti-inflammatory activity at 9.50 ± 4.89 μg/mL. Substitution pattern on the quinol nucleus is therefore essential since the *ortho* nitro group in compound **7** (1.22 ± 0.29 μg/mL) had the highest anti-inflammatory activity. The anti-inflammatory activity cannot always be correlated with antimicrobial activity, and this was observed for compound **10** which had good antifungal activity but least anti-inflammatory activity. In the same vein, the antioxidant activity did not always correlate with the anti-inflammatory activity but rather the antimicrobial activity. This was evident in compound **10** which demonstrated the highest antioxidant activity (Fig. 4) but the least anti-inflammatory activity and significant antimicrobial activity (Table 2 and Table 3). However, compound **7**, the highest anti-inflammatory *azo* compound, also exhibited good antifungal activity.

Nevertheless, further studies are necessary to ascertain the detailed mechanisms behind the anti-inflammatory activity of the *azo* compounds. They could be useful as lead compounds for developing agents for inflammatory diseases such as atherosclerosis, inflammatory bowel diseases, and rheumatoid arthritis.

3.5 Antioxidant activity

The ability of the *azo* compounds to donate hydrogen atoms or electrons were measured spectrophotometrically. The compounds reduced DPPH to the yellow-coloured diphenylpicryl hydrazine and diminished the absorbance at 517 nm, The free radical scavenging activities of the compounds are represented by their IC_{50} (50 % inhibitory concentration) as shown in Figure 4. The free radical scavenging activity of the *azo* compounds were studied by its capacity to decrease DPPH, a stable free radical, and any molecule that can donate hydrogen or electrons to the free radical decreases DPPH absorption intensity. This principle was demonstrated using ten *azo* compounds possessing catechol, naphtholic, and quinol backbones. These functional moieties are hydroxylated aromatics which can donate hydrogen or electrons to scavenge DPPH stable free radical, and this reaction is shown in Scheme 4.1.

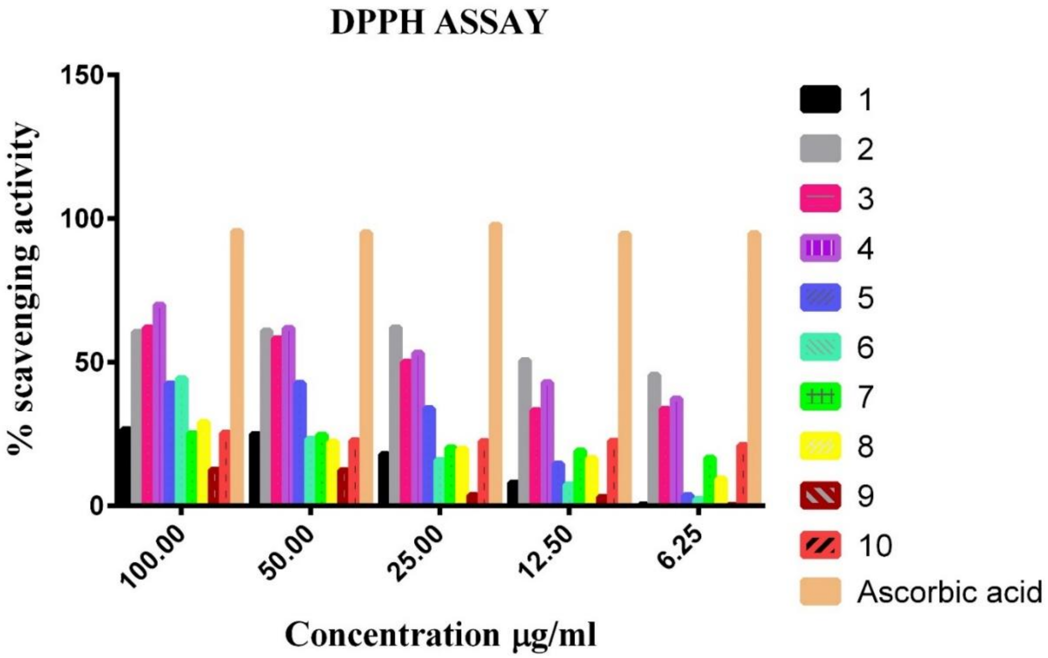


Figure 4: Scavenging activity (antioxidant activity) of the synthesized *azo* compounds [1-10]. Ascorbic acid [reference drug] μg/ml.

From the quantitative analysis of the DPPH radical scavenging activity of *azo* compounds (1-10), compound 10 was the most potent with an IC$_{50}$ of 1.00 ± 0.49 µg/mL. As a quinol derivative synthesised form *m*-nitro aniline, the two *p*-hydroxyl groups on the aromatic ring after donating protons to DPPH produced a phenoxide ion which is better stabilized by resonance than a monosubstituted hydroxyl group in the naphthol series. However, compound 2 possessing a *p*-ethylbenzoate group in the naphthol series exhibited an impressive antioxidant activity at 2.57 ± 0.70 µg/mL, suggesting the importance of electron-withdrawing groups in aiding DPPH free radical scavenging. It was also observed that electron-withdrawing nitro group in (compounds 7 [*o*-nitro, 3.881 ± 1.03 µg/mL] and 10 [*m*-nitro, 1.00 ± 0.49 µg/mL] was essential for antioxidant activity. The electron withdrawing groups through mesomeric effect create positive centres on the aromatic ring bearing the hydroxyl groups and these electron-deficient centres enhance the donation of protons to DPPH radical as to form the yellow product diphenyl-picrylhydrazine with decreased wavelength. This indicates the ability of these *azo* compounds possessing electron-withdrawing groups to scavenge DPPH free radicals effectively. The catechol series of compound 6 [o-nitro, 305 ± 177.72 µg/mL] and 9 [*m*-nitro, 86.57 ± 90.10 µg/mL had the least antioxidant activity. Though they possess electron-withdrawing nitro groups, the o-substituted hydroxyl groups diminished the antioxidant activity greatly. This could have been due to steric hindrance and intramolecular hydrogen bonding between the hydroxyl groups locking up the protons necessary for donation to DPPH radical. This could have prevented effective DPPH reaction with the catechol *azo* dyes hence decreasing the antioxidant activity. From the naphthol series, it was observed that compounds 2 [2.57 ± 0.70 µg/mL], 3 [8.56 ± 2.67 µg/mL] and 4 [7.27 ± 1.54 µg/mL] with ethylbenzoate, *p*-chloro and sulphonamide groups were the most potent free radical scavengers. It suggests that the presence of a nitro group in the naphtholic azo dyes reduced the antioxidant activity as seen in compounds 5 and 8, although the corresponding nitro derivatives had better potency in the catechol and quinol series. The presence of carboxylic acid group in compound 1 had an IC$_{50}$ of 40.6 ± 24.76 µg/mL than its naphtholic counterpart even though it had better antioxidant activity than compound 9, catechol [*m*-nitro] with an IC$_{50}$ of 86.57 ± 90.10 µg/mL.

Compound 10, a quinol nitro derivative, had the most impressive antioxidant activity at [1.00 ± 0.49 μg/mL] compared to the standard drug ascorbic acid [0.09 ± 0.12 μg/mL]. Considering its antimicrobial activity, it was the most active against resistant *Pseudomonas aeruginosa* and *Salmonella typhi* at 15.63 μg/mL. This suggests that there could be a possible correlation between antioxidant and antimicrobial activity. Moreover, compound 2 which also had a good antioxidant activity at 2.57 ± 0.70 μg/mL compared to ascorbic acid was active against *Escherichia coli* and *Streptococcus pyrogenes* at 62.5 μg/mL. It had MIC of 500 to >500 μg/mL against the rest of the microorganisms. This indicates that the antimicrobial activity cannot always be attributed to free radical scavenging in the microorganism. Although previous studies have demonstrated the strong antioxidant activity of *azo* compounds performed by using DPPH free radical scavenging assay, the mechanism of action of all the *azo* compounds would need to be investigated to make a strong conclusion on their antioxidant-antimicrobial correlation. However, the results of the antioxidant study show that these *azo* compounds may have a great significance in the therapy of diseases in which oxidative stress is implicated following lead optimisation.

4.0 Conclusions

A library of Ten (10) azo compounds were synthesised through the diazotisation and coupling pathways. Generally, the quinol and naphthol compounds had the best antimicrobial, anti-inflammatory, and antioxidant activities. The minimum inhibition concentrations of the most active compounds observed in this study showed that they have an inhibitory effects, thereby having a potential for use as medicines. The IC50's for the anti-inflammatory and antioxidant activity from this study is also an indication of their potential application in inflammation and oxidative stress experiments. However, evaluation of the effectiveness of the compounds in *the in vivo* experiments is recommended.

Data Availability

Data for this research is available at the Department of Pharmaceutical Chemistry and Molecular Biology lab at KNUST, Kumasi.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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