Original Research Article

4-hydroxyindole azo compounds demonstrate anti-biofilm formation activities against some pathogenic bacteria.

Comment [A1]: I suggest adding the bacterial strains to the title
For example: **4-hydroxyindole azo compounds demonstrate anti-biofilm formation activities against *Pseudomonas aeruginosas* and *Staphylococcus aureus***

Abstract

A hybrid of six (6) 4-hydroxyindole azo compounds was synthesised by the diazotization and coupling strategy *via* electrophilic aromatic substitution reaction. Characterisation by Infrared and UV-Vis spectroscopic studies was carried out and the antimicrobial activity and structure-activity relationship were explored. Amongst the compounds, 4d was the most active against *Pseudomonas aeruginosa* than the other organisms from the high-throughput spot culture growth inhibition (HTSPOTi) antimicrobial assay. According to the resistant modulation study, the compounds did not show significant activity against the panel of pathogens used. Furthermore, compounds 4a and 4f inhibited biofilm formation in *Pseudomonas aeruginosa* and *Staphylococcus aureus* at 70 % (31.25 µg/mL) and 57 % (125 (µg/mL) respectively. Additionally, 4c and 4e have biofilm inhibition potential against *Pseudomonas aeruginosa* and *Staphylococcus aureus* which are implicated in antimicrobial resistance. Hence, the compounds are promising leads with potential to be developed into new antibacterial agents to combat the menace of antimicrobial resistance.

Comment [A2]: Remove parentheses

Keywords: Synthesis, antimicrobial, azo compounds, pathogens.

Key Highlights

Comment [A3]: Describe the most important results of your work

- ~~Synthesis and antimicrobial studies of 4-hydroxyindole *azo* derivatives are described.~~
- Compounds 4c and 4e exhibited biofilm inhibition potential against *Pseudomonas aeruginosa* and *Staphylococcus aureus* ~~which are implicated in antimicrobial resistance.~~
- ~~The compounds provide structural scaffolds for lead optimisation in antimicrobial drug discovery.~~

1.0 Introduction

Infectious diseases according to the World Health Organisation are on the increase with the continuous rise in mortality cases every year over the world [1,2]. The burden of infectious diseases is a worrying situation since it continues to get worse due to the emergence of new resistant strains of pathogens as a result of polypharmacy, non-compliance to drugs and genetic mutations of the organisms over time [3]. The high incidence of nosocomial and community-acquired infections presents with critical challenges since they are usually associated with multi-drug resistant strains of *Pseudomonas*, *Acinetobacter*, *Staphylococcus*

and *Mycobacterium sp* [4]. In the case of *Mycobacterium sp*, which has one of the thickest biological membranes, the situation is alarming because of the emergence of multidrug and extensively drug-resistant strains which have become resistant to the first- and second-line antitubercular drugs due to different mechanisms such as genetic mutations, biofilm formation, quorum sensing and inherent efflux pumps [5]. Moreover, of major concern in the treatment of nosocomial infections are the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species* due to their resistant mechanisms [6]. Their presence in infections has been of public health concern since most of them are resistant to many antibiotics and appreciating the resistance mechanisms of these strains has been useful in the discovery of novel antimicrobial agents [7, 8]. However, in recent decades, the antibiotic reservoir is depleting, making the situation a worrying one [9, 10]. For instance, the discovery of only two novel classes of antibiotics in the last fifty years indicates that it may be a daunting task to develop enough classes of compounds to boost modern drug discovery in the future. Hence, strategies are required ahead of time to develop new therapeutic warheads that could either be used alone or as a combination therapy [11, 12].

Subsequently, azo dyes are libraries of suitable compounds to achieve this aim since they are an important class of antimicrobial candidates with versatile applications [13, 14]. It has been established that the inclusion of a suitable heterocyclic moiety enhances the activity of the azo linkage [14, 15]. However, the inclusion of nitrogen-containing aromatic heterocycles such as indole, quinoline, pyridine, thiadiazole and triazole confers biological activities including antimicrobial, anticancer, anti-inflammatory and anti-mycotic to the compound [16]. The indole nucleus is nowadays considered as an important moiety in the design and synthesis of bioactive compounds with antimicrobial, antioxidant, anti-inflammatory, antiviral and

anticancer effect [17]. Furthermore, there are review studies that indicate that in the field of pharmaceutical chemistry. Moreover, synthesis and full characterisation by spectral and thermal techniques have been reported in the literature for various derivatives bearing the indole nucleus [18]. The synthetic scheme of the azo dyes involves the diazotisation of primary aromatic amines and an electron rich moiety (coupling reagents) which mimics the principle of pharmacophore hybridisation [19, 20].

Following up on our previous studies, where we synthesised naptholic and phenolic azo dyes which demonstrated antimicrobial activity against some ESKAPE organisms, this present work sought to vary the heterocyclic nucleus in the construction of the azo dyes hinged on the importance of heterocyclics in antimicrobial drug discovery [21]. Hitherto, based on the success of previous studies, we set our goal to synthesise and evaluate the antimicrobial potential of six azo compounds against *Mycobacterium smegmatis* and some ESKAPE pathogens as a contribution to the drug discovery process.

2.0 Experimental

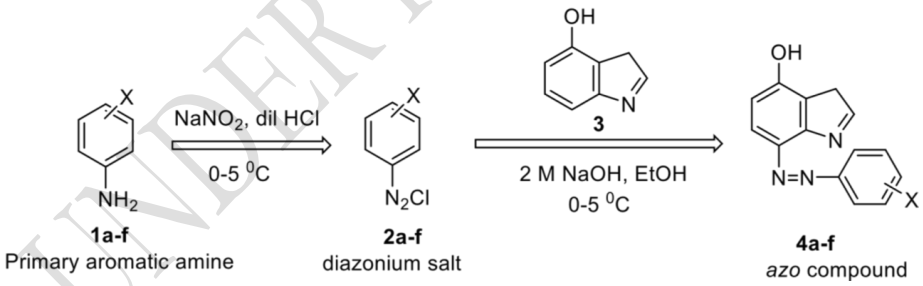
2.1 Chemicals, Reagents and Instrumentation

Reagents were obtained commercially from Fisher Scientific ™ (United Kingdom) and BDH chemicals (United Kingdom). The progress of the reactions was monitored using thin layer chromatography, which was performed by employing pre-coated silica gel plate (Merck F254) and visualised with UV light (254 nm and 357 nm). Compounds were purified by recrystallization from hot ethanol. Samples were run neat to obtain Infra-red spectra in the range 400–4000 cm^{-1} on a Bruker FTIR spectrophotometer. Ultraviolet-visible spectra were

obtained on a Shimadzu UV-Vis spectrophotometer at 200–800 nm with methanol as blank. Melting point data of the synthesised compounds were obtained by using one end or open capillary tubes on a Stuart melting point apparatus (England) and are uncorrected.

2.2 Synthesis and characterisation of azo compounds

The *azo* compounds were synthesised as previously described by dissolution of the appropriate primary aromatic amine (2 mmol) in dilute hydrochloric acid whilst keeping the reaction flask in a salt-ice bath (0-5 ^{o}C) until complete formation of the diazonium salt. This was followed by coupling with 4-hydroxyindole to obtain the crude product in yields of 65-95 %. The crude product was collected by suction filtration, washed and recrystallized from hot ethanol to give coloured solids **4a-f**.



Scheme 1: General scheme for the synthesis of the *azo* compounds **4a-f**; **4a** (X=*p*-NO_2), **4b** (X= *m*-NO_2), **4c** (X= *o*-NO_2), **4d** (X= 2-Cl-*p*-NO_2), **4e** (X=*p*-$COCH_3$), **4f** (X=*p*-COOH): *o*, *m* and *p* represent *ortho*, *meta* and *para* substitution respectively.

2.2.1 General procedure for synthesis and purification of the azo compounds [22]

Primary aromatic amine was reacted with $NaNO_2$ (1 M, 20 mL) in the presence of HCl (2 M, 20 mL, 36 % w/v) at a temperature range of 0-5 ^oC with the help of an ice bath to achieve diazotization. The clear diazonium solution is confirmed by the presence of an instantaneous blue colour on testing with starch iodide. Diazotized primary aromatic amine was treated with 4-hydroxyindole in the presence of NaOH (2 M, 200 mL, 98 % w/v) at a temperature range of 0-5 ^oC in an ice bath whilst stirring to achieve coupling. Precipitate of coupled compound was filtered under suction and dried at room temperature in a dessicator.

2.3 Antimicrobial Studies

2.3.1 Source of test organisms and materials

Standard reference strains *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), [Gram-negatives]. *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212) [Gram positives], *Mycobacterium smegmatis* mc^2 155 (ATCC 19420) [Acid-fast] were used in this study. The test organisms were obtained from the Cell Culture Laboratory, Department of Pharmacology, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana. The Gram positive and Gram-negative bacteria were cultured on nutrient agar while Middlebrook 7H10 agar supplemented with oleic acid, albumin, dextrose, and catalase (OADC) used for *Mycobacterium smegmatis* mc^2 155. Glycerol stocks of the pathogens were prepared and stored in -80 ^oC freezer for use.

Comment [A4]: I didn't understand the choice of *Mycobacterium* strain.
Why didn't you use *Mycobacterium tuberculosis*?

2.3.2 Culture media and reference antibiotics

Nutrient agar and Nutrient broth were purchased from Oxoid Limited, (Basingstoke, United Kingdom). Middlebrook 7H9 broth and Middlebrook 7H10 agar were purchased from Becton Dickinson and Company, Sparks, MD, USA. Ciprofloxacin and Rifampicin were obtained from Sigma Aldrich ™ (Michigan, USA). Tryptone Soy Broth (TSB) Sigma Aldrich ™ (Michigan, USA) was used for the biofilm assay.

2.3.3 Determination of Minimum Inhibitory Concentration (MIC) by High-throughput Spot Culture Growth Inhibition Assay (HT-SPOTi)

The experiment was carried out as described by Danquah *et al.,* (2016) [22]. Briefly, a stock concentration of the *azo* compounds was serially diluted using DMSO in a polymerase chain reaction (PCR) half-skirted 96-well plate to give a concentration range of 50 - 0.05 mg/mL. The DMSO was used at a final concentration of < 1% v/v. A volume of 2 µL of the *Azo* compounds were transferred into their corresponding wells in a standard 96-well plate, and then 200 µL of molten agar maintained at 55 - 60 °C was dispensed into each well with shaking to mix thoroughly. The plates were left undisturbed for the agar to solidify. Bacterial suspension (2 µL) of ~1×10^6 CFU/mL was spotted on each well. The plates were sealed with parafilm, wrapped with aluminum foil and incubated at a temperature of 37 °C for 18 to 24 h. Ciprofloxacin and Rifampicin were used as reference drugs for bacteria and *Mycobacterium* respectively. Wells with no drugs were included to serve as growth control. The 96-well plates were visually observed after incubation and the well containing the lowest

Comment [A5]: Did you use ciprofloxacin for Gram-positive bacteria?

concentration of a compound for which no growth was observed was determined as the minimum inhibitory concentration (MIC) in µg/mL of that compound against the organisms.

2.3.4 Resistant Modulatory Assay

To evaluate the effect of the most active compounds (4a [PNA], 4d [2Cl-4NA], 4e [$OCOCH_3$] and 4f [COOH]) on selected antibiotics, the combination assay of the compounds together with the antibiotic ciprofloxacin was evaluated using the HT-SPOTi assay with modifications [22 - 23]. The compounds were serially diluted in DMSO to include MIC and sub-MIC concentrations. The checker board was constructed by adding 1 µL of each of the dilution concentrations to the corresponding well and 1 µL of the MIC of the standard drug was added. The same procedure was carried out for the standard drug ciprofloxacin and the MIC's of the compounds added. A volume of 200µL nutrient agar medium or Middlebrook 7H10 agar medium supplemented with

Comment [A6]: Which antibiotics were used in the Resistant Modulatory Assay ? ciprofloxacin? Rifamicin ? or amoxicilina ?

OADC was dispensed *into the plates as previously described for bacteria and* *Mycobacterium* respectively. The plates were then spotted with 2 µL of bacteria (~ 1 x 10^6 CFU/mL), sealed and incubated at 37 ℃ for 24 h. The Fractional Inhibitory Concentration Indices (FICI) values were calculated using the formula:

$$\text{FICI} = \frac{\text{MIC}_\text{A} \text{ in the presence of B}}{\text{MIC}_\text{A}} + \frac{\text{MIC}_\text{B} \text{in the presence of A}}{\text{MIC}_\text{B}}$$

The combining effect of the componds in combination with ciprofloxacin or rifampicin against the pathogens was interpreted as follows: **Synergy, FICI ≤ 0.5; Indifference, 0.5 < FICI > 4.0; and antagonism, FICI ≥ 4.0** [24]

2.3.5 Biofilm Inhibition Assay

The ability of the compounds 4a, 4d, 4e and 4f to inhibit biofilm formation was measured using microplate-based assay described by [25]. The assay was performed in 96-well microtitre plates with some modifications. The bacterial cells were cultured in tryptone soy broth (TSB) supplemented with glucose to 1% w/v at 37 °C overnight. The compounds (4µL) were transferred into the microtitre plates from a two-fold serial dilution in TSB medium to give sub-MIC concentrations (1/2, 1/4, 1/8 and 1/16 of the MIC). The overnight culture was 1:100 diluted and 200 µL added to each well. The microtitre plates were incubated for 24 h at 37 ℃. After incubation, the broth was removed and the plates washed twice with phosphate buffered saline (PBS). The formed biofilms were heat-fixed by incubating at 60 ℃ for 30 minutes, stained with crystal violet 0.1 % (w/v) and excesses stain rinsed with distilled water. Biofilm formation was evaluated by adding 125 µL of 95 % v/v ethanol and acetic acid (3:1) to the wells, and the plates subjected to spectrophotometric reading at 600 nm using the microplate reader (Biotek Synergy H1 Hybrid Multi-Mode Reader: 271230). Wells containing bacteria and TSB and wells containing only TSB were included to serve as negative control and media control respectively. All tests were carried out in triplicate. The inhibitory activity was expressed as percentage biofilm inhibition using the equation:

$$Percentage\ Biofilm\ Inhibition\ (\%) = \left(\frac{Control\ OD_{600} - Test\ OD_{600}}{Control\ OD_{600}}\right) \times 100\%$$

The percentage of inhibition was plotted against the concentrations of the compounds using GraphPad Prism 6 (Figure SM1 and SM2).

Comment [A7]:
I can't see the figures!

2.4 Statistical Analysis

The results were analyzed using GraphPad Prism 6.0 software (GraphPad Software Inc.). The analysis of variance (ANOVA) followed by post-test Turkey or post-test Bonferroni was used to assess the differences between the groups. All the values are expressed as mean ± standard error of mean (SEM) from triplicate experiments.

3.0 Results and Discussion

3.1 Synthesis

All tested compounds (Table 1) were synthesised by our research group with yields ranging from 65- 95 %. This research includes the design and synthesis of analogues of 4-hydroxyindole azo compounds bearing benzene with different substituents (-NO_2, -COOH, -$COCH_3$). Diazonium salts of the primary aromatic amines were prepared by reaction of the substituted amines with sodium nitrite in the presence of hydrochloric acid. This reaction intermediate was then used to prepare the azo derivatives with their structural scaffold shown in Figure 1.

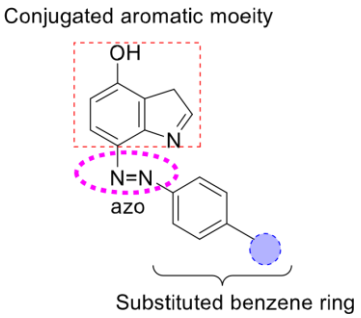


Fig. 1: Scaffold design for azo compounds **4a-f**

The synthesis of the library of azo dyes **(4a-f)** was performed according to methods. The compounds were obtained in good yields and high purity confirmed by TLC, melting point and spectroscopic methods. The physical properties (Table 1), and UV-Vis transitions (Table 2) of the azo compounds have been shown from the analysis. The absorption curves (200-400 nm) in methanol are shown in Figure 2 and the various transitions of the compounds are shown in Table 2.

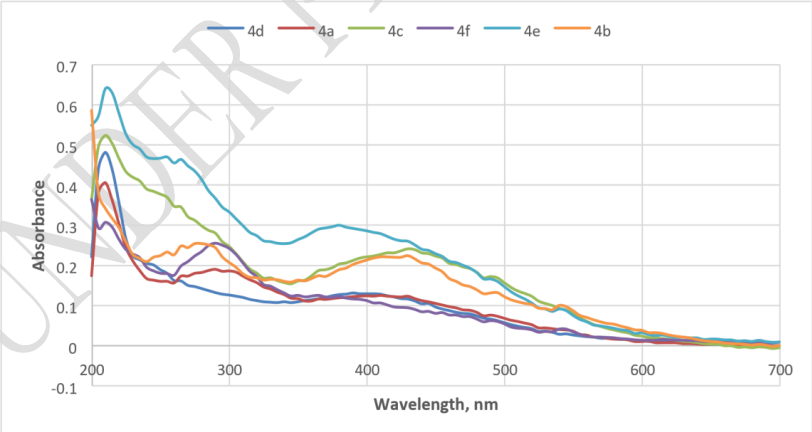


Fig. 2: UV-Visible curves of compounds (4a-f)

Table 1: Some Physical properties of the azo compounds

Compound	R	% yield	Melting points (^{o}C)	colour
4a	*p*-nitro	95	287-300	Dark brown
4b	*m*-nitro	82	224-227	Dark brown
4c	*o*-nitro	78	218-221	Pale brown
4d	*o-chloro-nitro*	65	290-293	Pale brown
4e	*p*-$COCH_3$	72	220-223	Dark brown

4f	*p*-COOH	95	290-292	Dark brown

Table 2: Types of UV-Visible transition for the azo compounds

Compound	Type of transition	Wavelength (nm)
4a	$\pi \rightarrow \pi^*$, $n \rightarrow \pi^*$ *and* $\pi \rightarrow \pi^*$	260 (sharp), 290(shoulder) and 420 (tautomerism)
4b	$\pi \rightarrow \pi^*$, $n \rightarrow \pi^*$ *and* $\pi \rightarrow \pi^*$	280 (sharp), 345 (shoulder) and 430 (tautomerism)
4c	$\pi \rightarrow \pi^*$, $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$	230 (sharp), 345 (shoulder) and 430

		(tautomerism)
4d	$\pi \rightarrow \pi^*$, $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$	240 (sharp), 345 (shoulder) and 390 (tautomerism
4e	$\pi \rightarrow \pi^*$, $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$	260 (sharp), 290 (shoulder) and 410 (tautomerism)

Synthetic data on the *azo* compounds

Compound 4a

According to the general procedure, *p*-nitroaniline (1.04 g) was reacted with $NaNO_2$ (1 M, 20 ml) in the presence of HCl (2 M, 20 mL, 36 % w/v) to afford the *title compound* (95 %w/w) as a dark brown solid after purification (R_f 0.78; EtOAc: MeOH (9:1); Mpt: 287-300 ^oC; UV-Vis/ nm: 260, 290 and 420; IR ν_{max}/cm^{-1}: 3380 (O-H), 1492 (N=N), 1244 (C-N) stretch.

Compound 4b

According to the general procedure, *m*-nitroaniline (1.04 g) was reacted with $NaNO_2$ (1 M, 20 ml,) in the presence of HCl (2 M, 20 mL, 36 % w/v) to afford the *title compound* (82 %w/w) as a dark brown solid after purification (R_f 0.76; EtOAc: MeOH (9:1); Mpt: 224-227 ^oC; UV-Vis/ nm: 280, 345 and 430, IR ν_{max}/cm^{-1}: 3375 (O-H), 1489 (N=N), 1256 (C-N) stretch.

Compound 4c

According to the general procedure, *o*-nitroaniline (1.04 g) was reacted with $NaNO_2$ (1 M, 20 ml,) in the presence of HCl (2 M, 20 mL, 36 %) to afford the *title compound* (78 %w/w) as an pale brown solid after purification (R_f 0.75; EtOAc: MeOH (9:1); Mpt: 218-221 ^oC; UV-Vis/ nm: 230, 345 and 430; IR ν_{max}/cm^{-1}: 3375 (O-H), 1433 (N=N), 1340 (C-N) stretch.

Compound 4d

According to the general procedure, *p*-aminobenzoic acid (1.04 g) was reacted with $NaNO_2$ (1 M, 20 ml,) in the presence of HCl (2 M, 20 mL, 36 % w/v) to afford the *title compound* (60 % w/w) as a pale brown solid after purification (R_f 0.80; EtOAc: MeOH (9:1); Mpt: 290-295 ^oC; UV-Vis/ nm: 240, 345 and 390; IR v_{max}/cm^{-1}: 3066 (O-H), 1488 (N=N), 1312 (C-N) stretch.

Compound 4e

According to the general procedure, *p*-aminoacetophenone (1.04 g) was reacted with $NaNO_2$ (1 M, 20 ml,) in the presence of HCl (2 M, 20 ml, 36 % w/v) to afford the *title compound* (72 % w/w) as a dark brown solid after purification (R_f 0.70; EtOAc: MeOH (9:1); Mpt: 220-223 ^oC; UV-Vis/ nm: 240, 340 and 380; IR v_{max}/cm^{-1}: 3378 (O-H), 1492 (N=N), 1357 (C-N) stretch.

Compound 4f

According to the general procedure, *p*-aminobenzoic acid (1.04 g) was reacted with $NaNO_2$ (1 M, 20 ml,) in the presence of HCl (2 M, 20 mL 36 % w/v) to afford the *title compound* (65 % w/w) as a pale brown solid after purification (R_f 0.65; EtOAc: MeOH (9:1); Mpt: 290-292 ^oC; UV-Vis/ nm: 260 and 290; IR v_{max}/cm^{-1}: 3090 (O-H), 1472 (N=N), 1330 (C-N) stretch.

3.2 Antimicrobial studies

The antimicrobial studies included determination of the minimum inhibitory concentration, resistant modulation and biofilm inhibition.

3.2.1 Minimum inhibitory concentration (MIC)

The antimicrobial study is represented by the minimum inhibitory concentrations of the azo compounds (Table 3).

Table 3: Antimicrobial activity of the synthesized azo compounds

	Minimum Inhibitory Concentrations (µg/mL)				
Compounds	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Enterococcus faecalis*	*Escherichia coli*	*Mycobacterium smegmatis*
4a	125	250	>500	500	>500
4b	>500	250	>500	500	>500
4c	250	250	>500	250	>500
4d	62.5	250	>500	>500	>500
4e	125	500	>500	500	>500
4f	125	250	250	500	>500
Ciprofloxacin	0.008	1.95	10	1.95	≤ 0.5
Rifampicin	nd	nd	nd	nd	1.95

nd: Not determined for the ESKAPE organisms used for the antimicrobial activity.

3.2.1.1 Structure-Activity relationship effect on MIC

Comment [A8]: What references support your assumptions?

It was observed that the azo compounds exhibited better antimicrobial activity against Gram-negative *Pseudomonas aeruginosa* than the other organisms. The best activity against *Pseudomonas aeruginosa* was for 4d which has the chloro and nitro group *ortho* and *para* positions to the azo bond respectively. The least activity was observed generally for the compounds against all the organisms except *Pseudomonas aeruginosa.* This suggests that the presence of a chloro and nitro groups at ortho and para positions to the azo bond is essential for antipseudomonal activity but not relevant against the other pathogens. The extra chloro group could have increased the lipophilicity of 4d to penetrate the membranes of the organism compared to other compounds which possess only a nitro group or a ketone functionality as in 4f. The study also revealed that the nitro, carboxylic acid and ketone moieties individually did not affect the antimicrobial activities of the compounds against *Mycobacterium smegmatis.*

3.2.2 Resistant Modulatory Activity

One effective strategy required to win the battle against antimicrobial resistance is combination therapy, as it offers an advantage of modulation of the activities of already

available antimicrobials by promising lead compounds through a possible reversal of resistance mechanisms by causative organisms [26]. For infections including human immunodeficiency virus and tuberculosis, combination therapy is considered as a standard to achieve treatment outcomes. Though, combination therapy is an effective strategy, some reports on their therapeutic effects to prevent resistance are conflicting and need to be investigated by establishing the fractional inhibitory concentration index for the potential modulating agents [27]. The fractional inhibitory concentration index (FICI) is a quantitative measure of resistant modulatory activity**.** The FICI method is reported as one of the best combination methods used to determine the impact on potency of the combination of antibiotics when compared to their individual activities [27]. The combinatory effect of the compounds P-NA (4a), 2Cl-NA (4d), 4AA (4e), and PABA (4f) was examined in combination with the antibiotic amoxicillin against *P. aeruginosa* and *S. aureus* (Table 4).

Table 4: Resistant modulatory activity of the most active compounds

Compound	FICI	
	P. aeruginosa	*S. aureus*
Amoxicillin + 4a	66.10 (AT)	8.12 (AT)
Amoxicillin + 4d	1.5 (NI)	8.14 (AT)
Amoxicillin + 4f	4.35 (AT)	1.06 (NI)
Amoxicillin + 4e	0.75 (NI)	2.13 (NI)

Fractional Inhibitory Concentration Indices of the combination of the compounds and Amoxicillin: Synergistic effect expressed as the fractional inhibition concentration index (FICI), calculated from the MIC of the various compound/antimicrobial combinations. The FIC index interactions were defined as: FICI ≤ 0.5, (S) synergy; >0.5 to ≤4.0 (NI) no interaction; and >4.0, (AT) antagonistic.

From Table 4, a reduction in activity in the combination of 4a/ Amoxicillin against both *P. aeruginosa* and *S. aureus* strain was observed. The FIC index result showed an antagonistic interaction when 4a was combined with the antibiotic. A similar result was observed in the 4d/ Amoxicillin combination, with no interaction between the compounds in both bacterial strains. However, in the combinatory effect of the antibiotic with 4d, there was no interaction against *P. aeruginosa* but an antagonism effect was observed with *S. aureus*. Compound 4f exhibited antagonism in *P. aeruginosa* and showed no difference in effect against *S. aureus* when combined with amoxicillin. Hence, in this study, none of the compounds demonstrated synergistic activity with amoxicillin against both strains of bacteria used. The interaction observed was either antagonism or there was no effect. The results suggest that the compounds 4a, 4d, 4f and 4e do not interact synergistically with amoxicillin against *P. aeruginosa* and *S. aureus*, hence, may not be used as promising synergistic agent. Furthermore, to reduce the toxic side effects of other antibiotics by reducing their dose, studies should be conducted with other standard antibiotics to check the compounds for synergistic effect since they have shown promising antimicrobial effect. A good synergistic effect suggests the potential to combine these compounds with standard antibiotics such that

their doses can be reduced and the manifestation of their toxic side effects can subsequently be reduced [28].

3.2.3 Biofilm Inhibition Assay

Bacterial biofilms are highly organized bacterial complexes encased in a self-produced matrix protected from the host defense [29 - 30]. They enhance reduced antibiotic susceptibility hence, contributes to the persistence of biofilm infections. Compared to their planktonic forms, biofilms are known to have high adaptive resistance to antibiotics and other disinfectants. This makes treating biofilm-associated infections very difficult [30]. In addition, bacterial biofilms in *P. aeruginosa* and *S. aureus* are increasingly recognized as an important virulence factor contributing to the persistence of biofilm associated infections and multidrug resistance. Therefore, it has become necessary to find effective strategies to control and eliminate these biofilms in the fight against biofilm related infections. The most active compounds from previous studies were investigated for their ability to inhibit biofilms at sub-inhibitory concentrations against *P. aeruginosa* and *S. aureus*. The results of the biofilm inhibition of the compounds are shown in Figure SM1 and Figure SM2.

Regarding the biofilm inhibitory activities of the *azo* compounds, all the compounds exhibited marked activity at sub-inhibitory concentrations. The compounds showed variable effects on the inhibition of biofilms in the strains used. The inhibitory activity against the *S. aureus* biofilms was in a concentration dependent manner for all the compounds. Generally, 4a and 4d had very good inhibitory effect in all the concentrations used. At 1/16 MIC, the compounds still had biofilm inhibitory effect >20 %. However, the biofilm inhibitory activity against *P. aeruginosa* also showed concentration dependent effect with the lower

concentrations showing higher activity [Figure SM2]. Compound 4f had the most significant inhibitory effect in all the concentrations (<60 % inhibition). The compounds however, showed different susceptibility patterns in *P. aeruginosa* and *S. aureus.* (Figure SM1 and SM2). The effects of the compounds against *S. aureus* biofilms was concentration-dependent with 4a at 1/2 MIC (125 µg/mL) giving the highest effect (57.33 %). The *azo* compound 4e which showed a higher MIC compared to 4d and 4f, gave a more significant biofilm inhibitory effect than 4d, 4f and 4a (except at 1/2 MIC). Thus, though 4a exhibited little inhibition against planktonic growth of *S. aureus*, it however demonstrated significant biofilm inhibitory activity compared to 4d and 4f. *Staphylococcus aureus* biofilms have become recalcitrant to antimicrobial treatment and the host response, and therefore are the etiological agent of many recurrent infections especially nosocomial infections and chronic wounds. Biofilm inhibition increased with decreasing concentration of the compounds in *P. aeruginosa*. Compound 4f showed the highest biofilm inhibition in all the concentrations used with the highest effect (70 %) at 1/16 MIC (31.25 µg/mL). The compounds 4e, 4d, and 4a continued in that manner. The *azo* compound 4d which showed good antimicrobial activity against *P. aeruginosa*, exhibited a minimal biofilm inhibition compared to 4d and 4a. The presence of the carboxylic acid group at the *para* position of the aromatic ring increased the antimicrobial, resistant modulatory and biofilm inhibition against *S. aureus* and *P. aeruginosa*. The presence of the ketone group followed by the 2-chloro-4-nitro and the *p*-nitro moieties in the order influenced the biofilm activity greatly. The biofilm inhibition effect of the azo compounds validates the fact that there is no strict pattern with regards to effects of sub-inhibitory concentrations of antibiotic compounds on biofilm inhibition. Hence, biofilm inhibition in different bacterial species is based on specificity of the response to sub-inhibitory concentrations of the compounds.

Conclusion

A library of six compounds was successfully synthesised from diazotisation and coupling procedures. Compound 4d was the most active with a minimum inhibitory of 62.5 µg/mL concentration but against against *Pseudomonas aeruginosa* using the high-throughput spot culture growth inhibition (HT-SPOTi) antimicrobial assay. Compounds 4a and 4f inhibited biofilm formation in *Pseudomonas aeruginosa* and *Staphylococcus aureus* at 31.25 µg/mL and 125 µg/mL respectively. Compounds 4c and 4e exhibited biofilm inhibition potential against *Pseudomonas aeruginosa* and *Staphylococcus aureus* which are implicated in antimicrobial resistance. These compounds could serve as starting point for detailed structure elucidation and optimisation.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement

of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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