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IN VITRO ANTIBACTERIAL AND SYNERGISTIC ACTIVITY OF PYRAZOLYL SULPHONAMIDE DERIVATIVES AGAINST STAPHYLOCOCCUS HAEMOLYTICUS

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ABSTRACT
Background: Antimicrobial resistance has rendered several anti-infective agents ineffective, necessitating the need to intensify efforts to identify and develop novel drugs against microbial infection. Structural modification of existing antibiotics continues to be one of the areas of intense research focus in recent times. **Objective:** This study assessed the antibacterial and synergistic activity of fifteen pyrazolyl sulphonamide derivatives in *Staphylococcus haemolyticus*. **Methods:** Antibacterial activity was determined using the broth microdilution method. The ability of test compounds to interact with tetracycline was assessed using the checkerboard synergy testing method and the Loewe synergy and antagonism model. **Results:** Seven compounds (46.6%) were significantly active against the bacteria (MIC 1 µg/mL – 16 µg/mL), and four were confirmed to form synergistic combinations with tetracycline in checkerboard and Loewe analysis. **Conclusion:** Observations from this study has demonstrated the antibacterial activity and the synergistic potential of the novel pyrazolyl sulphonamides with tetracycline, highlighting the possible role of modified sulphonamides as a rich resource for antimicrobial development.

INTRODUCTION

Staphylococcus haemolyticus is a coagulase-negative staphylococci (CNeS) that is commonly found on the skin and often associated with infections, particularly in hospital settings [1]. It has gained clinical relevance due to its ability to cause nosocomial infections, especially in patients undergoing chemotherapy, organ transplantation, HIV/AIDS, and other immunocompromised individuals or those with underlying health conditions. As a common micro-organism found in healthcare environments, *S. haemolyticus* is often associated with indwelling medical devices such as catheters, prosthetic implants, and other devices, making it a significant cause of nosocomial infections [1].

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For many years, *S. haemolyticus* was considered an apathogenic commensal in the human skin and mucosa because the virulence attributes associated with other staphylococci, such as *S. aureus*, were not observed with it. This was due mainly to our inability to accurately distinguish between species of this genus because of their seemingly close genetic relatedness [2, 3]. However, with the introduction of more sophisticated biomedical procedures like molecular techniques, the role of this species in diverse clinical infections has become more evident. Pinheiro and colleagues detected and characterized various enterotoxin and cytotoxin genes from *S. epidermidis* and *S. haemolyticus* isolated from blood cultures [3]. Enterotoxins stimulate the immune system, causing an exaggerated release of proinflammatory cytokines, which account for the rapid onset of high fever and multiple organ dysfunction. This could partly account for the ability of the CNeS to cause numerous human infections, such as skin and soft tissue infections, especially in the elderly and immunocompromised individuals.

This bacterial species is currently known to be associated with multiple opportunistic infections of clinical relevance. *Staphylococcus haemolyticus* is linked to various infections, such as peritonitis, meningitis, urinary tract infections, and otitis. It is among the most frequently isolated CNeS in patients with urinary tract and blood infections like sepsis [4]. Infections caused by this organism are often treated with ciprofloxacin, clindamycin, erythromycin, gentamicin, vancomycin, and penicillins [5]. Although antibiotics abound for the management of *S. haemolyticus* and other infections, the increasing incidence of resistance of this organism to antimicrobial therapy has become a significant interest of public health concern [1]. Antimicrobial resistance, a phenomenon where infecting organisms can adapt and grow in the presence of previously effective antimicrobial agents, is essentially a result of (i) the inability of patients to adhere to prescription instructions, (ii) weak pharmaceutical regulations in many parts of the world, and (iii) improper use within agricultural settings [6]. These factors facilitate the development and spread of antimicrobial-resistant organisms, making treatment of primary infectious conditions difficult and more expensive, resulting in increased infection-related morbidity and mortality. Although much has been done to reduce inappropriate prescriptions, enhance patient compliance, ensure appropriate use of antimicrobials in farming, boost activities of regulatory agencies, and, in some instances, use of combination drug therapy, resistant strains still emerge rapidly [6, 7]. The slow progress in our attempt to develop new and more effective antimicrobials to combat microbial threats is coupled with this threat of AMR.

Structural modification of the chemical structures of existing agents is one of the areas of intense research. This approach allows for introducing various chemical groups into the core pharmacophore of an antimicrobial agent without altering the components required for microbial activity [8, 9]. In the first half of the nineteenth century, the discovery of prontosil, a sulphonamide, gave life to man's attempt to overcome microbial infections. Since then, several other antimicrobial medications have been derived from the structural modification of the core sulphonamide structure to enhance treatment outcomes. The emergence of sulphonamide-resistant microorganisms has diminished the clinical usefulness of many of these sulphonamides, hence the need to find more effective compounds. The current study evaluated pyrazolyl sulphonamide derivatives' antibacterial and synergistic activity against *S. haemolyticus*.

MATERIAL AND METHODS

Ethical Approval

The study was approved by the ethics committee of the Korle Bu Teaching Hospital (KBTH-IRB/000106/2018).

Test microorganism

Staphylococcus haemolyticus (042W_ 91123) was isolated from wounds of burn patients admitted at the Burns Unit of Korle Bu Teaching Hospital from January to December 2023. The samples were cultured on MacConkey agar (Oxoid Ltd, Basingstoke, United Kingdom) and incubated at 37°C for 18 hours. Pure colonies were further identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) with a Microflex LT Biotyper 3.0 (Bruker, Daltonics, Bremen, Germany) by the manufacturer's instructions.

Chemicals and Reagents

The chemicals and reagents used were MacConkey agar and Mueller Hinton broth from Oxoid Ltd (Basingstoke, UK), Tetracycline hydrochloride from Cayman Chemical (USA), and Dimethylsulfoxide (DMSO) from Sigma-Aldrich (USA). The Chemistry Department, University of Ghana synthesized the pyrazolyl sulphonamide derivatives. All compounds were characterized as previously reported [10].

Pyrazolyl Sulphonamide derivatives

A total of fifteen sulphonamide derivatives consisting of four single sulfonated phenyl pyrazolyl sulphonamides, four double sulfonated phenyl pyrazolyl sulphonamides, three phenyl-sulfonated pyrazolyl sulphonamides, and four single sulfonated phenyl pyrazolyl sulphonamide palladium (II) complexes were tested in this study. All compounds were synthesized by a multi-step procedure and characterized by nuclear magnetic resonance spectroscopy, infrared spectroscopy, electrospray ionization mass spectrometry, and in some instances by single X-ray crystallography as described earlier [10]. Stock solutions of all test samples were prepared by dissolving in 0.5% DMSO (Sigma-Aldrich, USA) solution and stored at -20°C in closed containers until use. Figure 1 shows the structures of the pyrazolyl sulphonamide compounds used in this study.

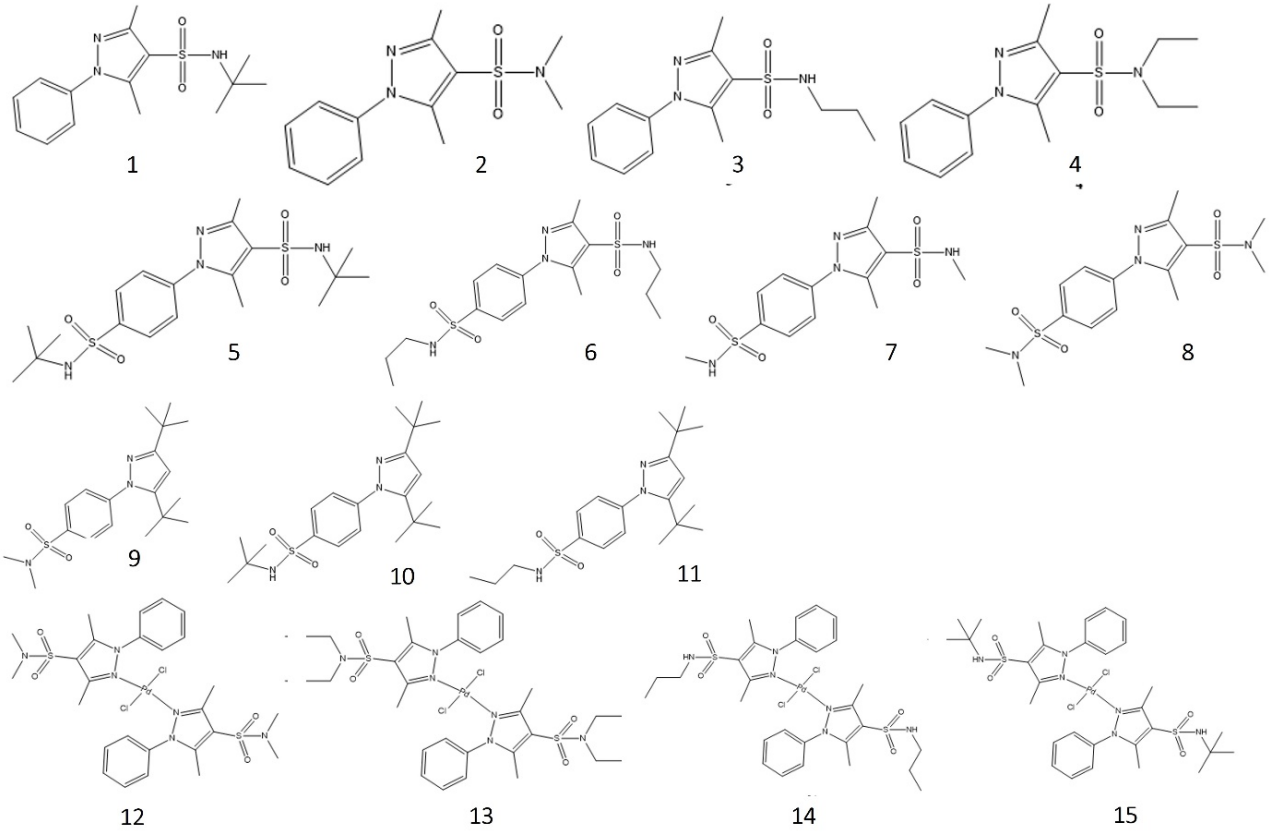

Figure 1: Structural identity of pyrazolyl sulphonamide derivatives tested against selected Gram-positive bacteria. (1) N-(tert-butyl)-3,5-dimethyl-1-phenyl-1H-pyrazole-4-sulfonamide; (2) N,N,3,5-tetramethyl-1-phenyl-1H-pyrazole-4-sulfonamide; (3) 3,5-dimethyl-1-phenyl-N-propyl-1H-pyrazole-4-sulfonamide; (4) N,N-dimethyl-3,5-dimethyl-1-phenyl-1H-pyrazole-4-sulfonamide; (5) N-(tert-butyl)-1-(4-(N-(tert-butyl)sulfamoyl)phenyl)-3,5-dimethyl-1H-pyrazole-4-sulfonamide; (6) 3,5-dimethyl-N-propyl-1-(4-(N-propylsulfamoyl)phenyl)-1H-pyrazole-4-sulfonamide; (7) N,3,5-trimethyl-1-(4-(N-methylsulfamoyl)phenyl)-1H-pyrazole-4-sulfonamide; (8) 1-(4-(N,N-dimethylsulfamoyl)phenyl)-N,N,3,5-tetramethyl-1H-pyrazole-4-sulfonamide; (9) 4-(3,5-di-tert-butyl-1H-pyrazole-1-yl)-N,N-dimethylbenzenesulfonamide; (10) N-(tert-butyl)-4-(3,5-di-tert-butyl-1H-pyrazole-1-yl)benzenesulfonamide; (11) 4-(3,5-di-tert-butyl-1H-pyrazole-1-yl)-N-propylbenzenesulfonamide; (12) Dichloro[bis(N,N,3,5-tetramethyl-1-phenyl-1H-pyrazole-4-sulfonamide)]palladium(II); (13) Dichloro[bis(N,N-dimethyl-3,5-dimethyl-1-phenyl-1H-pyrazole-4-sulfonamide)]palladium(II); (14) Dichloro[bis(3,5-dimethyl-1-phenyl-N-propyl-1H-pyrazole-4-sulfonamide)]palladium(II); (15) Dichloro[bis(N-(tert-butyl)-3,5-dimethyl-1-phenyl-1H-pyrazole-4-sulfonamide)]palladium(II)

Determination of the Antibacterial Activity of Compounds

A preliminary antibacterial activity of the compounds using the disk diffusion method was reported earlier [10]. The standard broth dilution method [11, 12] was used to study the antibacterial activity of the compounds against isolates of *S. haemolyticus* (042W_91123) by evaluating the visible growth of

microorganisms in nutrient broth (Oxoid Ltd, Basingstoke, UK). Two-fold serial dilutions of test compounds in concentrations ranging from 0.0625 µg/mL to 64 µg/mL with adjusted bacterial concentration (10^8 CFU/mL, 0.5 McFarland's standard) in 96-well plates were used to determine the minimum inhibitory concentration (MIC). The positive control wells were prepared similarly, having tetracycline (0.0625 µg/mL – 16 µg/mL) instead of test compounds. Negative control wells contained only inoculated broth. Plates were incubated at 37°C for 24 hours, and optical density was determined using the spark multimode microplate reader (Tecan Spark, V3.2, Switzerland) at 600 nm.

Determination of the Synergistic Activity of Compounds

The interactive effect of combining the test pyrazolyl derivatives and tetracycline was determined using the Checkerboard Synergy Testing method in 96-well plates as described previously [13]. Working solutions of each test compound were prepared in nutrient broth as indicated above and serially diluted to ten different concentrations (0.0625 µg/mL – 32 µg/mL) in microtitre plates in duplicates. The tetracycline antibiotic (Cayman Chemical, USA), which served as the positive control, was similarly prepared and serially diluted to seven different concentrations ranging from 0.0625 µg/mL to 4 µg/mL. Compounds were tested alone and in combination with tetracycline. Plates were incubated at 37°C for 24 hours, and optical density was determined using the spark multimode microplate reader (Tecan Spark, V3.2, Switzerland) at 600 nm. The interpretation of the fractional inhibitory concentration (FIC) was applied as follows: synergy, ≤ 0.5; indifference, > 0.5 to ≤ 4.0; antagonism, > 4.0 [13, 14].

The FIC index (FICI) was calculated using the formula:

$$FICI = \frac{MIC^{a2}}{MIC^{a1}} + \frac{MIC^{b2}}{MIC^{b1}}$$

Where, MIC^{a1} = MIC of test compound; MIC^{a2} = MIC of test compound in combination; MIC^{b1} = MIC of positive control; MIC^{b2} = MIC of positive control in combination.

To confirm the nature of the interaction, single dose-effect and combination dose-effect analyses were conducted using the Loewe synergy and antagonism model as described previously [14, 15].

Statistical Analysis

Each data represents an average of three independent experiments. The data was analyzed by one-way ANOVA followed by Tukey's post hoc test using the GraphPad Prism. Statistical significance was considered at $p < 0.05$.

RESULTS AND DISCUSSION

Sulphonamides were among the first agents used to manage bacterial infections. However, with the emergence of bacterial resistance to this group of antimicrobials, their clinical use has seen a downturn [16, 17]. Globally, immense effort has been invested in research to discover novel antimicrobial compounds. In this current study, the ability of fifteen novel pyrazolyl sulphonamide compounds to terminate or inhibit the growth of *S. haemolyticus* was evaluated by determining the MICs of each compound.

Except for the palladium-complexed sulphonamide compounds, all other groups of sulphonamides tested yielded activity against *S. haemolyticus*. The single sulfonated phenylpyrazole sulphonamides (Compounds 1 – 4) were the most active of the tested sulphonamides, with ¾ of the tested compounds being active against the bacteria (Table 1). Compounds 1 and 4 (C1 and C4, MIC = 2 µg/mL) were four times more potent than C2. On the other hand, half of the double and phenyl sulfonated sulphonamides demonstrated activity against the bacteria, with MICs ranging from 1 µg/mL to 16 µg/mL. With an MIC of 1 µg/mL, C10 was closest in activity to the tetracycline antibiotic (MIC = 0.25 µg/mL). Generally, sulphonamide compounds produce antibacterial effects by inhibiting the bacterial synthesis of folic acid, a pivotal component required for replicating the organism's DNA [18].

The structural similarity between sulphonamides and *p*-aminobenzoic acid (PABA), a precursor for folic acid synthesis, enables this group of antimicrobials to inhibit and replace endogenous PABA and consequently terminate DNA synthesis. Hence, the effect of the novel sulphonamides against *S. haemolyticus* could result from the ability of the compound to inhibit bacterial folic acid and DNA synthesis. On the other hand, the increasing frequency of multi-drug resistant strains of *S. haemolyticus* is already well known. Resistance to the bacteria has been reported in significant antibiotics of clinical importance, including the β-lactam antibiotics, vancomycin, methicillin, and mupirocin [1]. This widespread resistance could explain the lack of activity associated with more than half of the tested sulphonamides in this study. However, the bacteria's susceptibility to seven compounds illustrates the potential of the

sulphonamide derivatives as a source of potent antibiotics against *S. haemolyticus* infections.

Furthermore, a single dose-response analysis of the compounds confirmed their potential antibacterial effect (Table 1). The concentration of compounds capable of inhibiting the growth of the bacteria by 50% (EC_{50}) within the tested period ranged from 0.8 µg/mL to 11.5 µg/mL, compared to 0.2 µg/mL for the tetracycline antibiotic. This confirms the potency of the compounds against *S. haemolyticus*, with C10 being the most potent and C6 being the least powerful, as earlier illustrated.

All sulphonamide antibiotics act by competitively inhibiting bacterial folic acid synthesis and preventing the replication and formation of DNA and essential proteins [18]. The differential activity of the test compounds against the bacteria could be due to unequal affinity for the bacteria's active site. This may result from many factors, including compound lipophilicity differences [18, 19].

Table 1: Antibacterial and fractional inhibitory indices of pyrazolyl sulphonamide compounds in *S. haemolyticus*

Compound ID	EC_{50} (µg/mL)	MIC alone (µg/mL)		MIC in combination (µg/mL)		FICI	Interaction pattern
		S^a	T^a	S^b	T^b		
1	1.4	2	0.25	0.0625	0.0625	0.2813	Synergistic
2	9.2	8	0.25	0.0625	0.5	2.0078	Indifferent
4	1.8	2	0.25	0.0625	0.0625	0.2813	Synergistic
5	1.6	2	0.25	0.0625	1	4.0313	Antagonistic
6	11.5	16	0.25	0.0625	0.125	0.5039	Indifferent
10	0.8	1	0.25	0.0625	0.0625	0.3125	Synergistic
11	2.4	2	0.25	0.0625	0.0625	0.2813	Synergistic

EC_{50}, Half maximal effective concentration; FICI, Fractional inhibitory concentration; MIC, Minimum inhibitory concentration; S^a, MIC of test sample alone; S^b, MIC of test sample in combination; T^a, MIC of tetracycline alone; T^b, MIC of tetracycline in combination

Synergistic Activity

Combination therapy has become a standard practice in many areas of modern medicine, such as antimicrobial, anticancer, and antihypertensive therapies, because of the enhanced therapeutic benefit. Hence, it is important to provide evidence of drug combinations' superiority over monotherapy. Using the checkerboard method, this study demonstrated the potential synergistic activity of sulphonamide-tetracycline combinations. The checkerboard method remains the most reliable method for determining the synergistic activity of antimicrobials. However, it is fraught with limitations like the lack of standardization in interpreting results and reduced precision at higher concentrations of test compounds [20]. Results are expressed mathematically as the fractional inhibitory concentration (FIC), which combines the respective ratios of the concentration of drugs in combination wells with their MICs. Table 1 shows the outcomes of the different compound-tetracycline combinations evaluated for active sulphonamide derivatives.

Synergism was demonstrated in tetracycline combinations involving C1, C4, C10 and C11. No interaction was observed for two compound combinations (C2 and C6), but C5 was found to antagonize the antibiotic effect of tetracycline in *S. haemolyticus*. Bacterial resistance to the sulphonamide antibiotics makes their continuous use as monotherapy ineffective. Hence, their combination with other antibiotics, especially those with a different mechanism of action, like tetracyclines, could be clinically helpful. Tetracycline combination therapy is already being utilized in the clinical management of Gram-positive infections, among others [21]. The synergistic activity exhibited by four compounds tested in this study indicates their potential usefulness and should be explored further.

Loewe Synergy and Antagonism analysis

The Loewe Synergy and Antagonism Model is a widely used framework for assessing the interaction between two or more compounds, thereby enabling the identification of combinations with enhanced therapeutic benefits or unwanted effects [9, 13, 14]. It helps determine whether the combined effect of drugs is synergistic, antagonistic, or additive. The model is based on the idea that if two or more drugs act independently on the same

biological system, their combined effect can be predicted by the sum of their individual effects. The effect is additive when the outcome of a drug combination matches the expected impact from their independent actions. Synergy occurs when the drugs enhance each other's efficacy beyond what is expected from their actions. On the other hand, antagonism occurs if the presence of one drug reduces the effectiveness of the other.

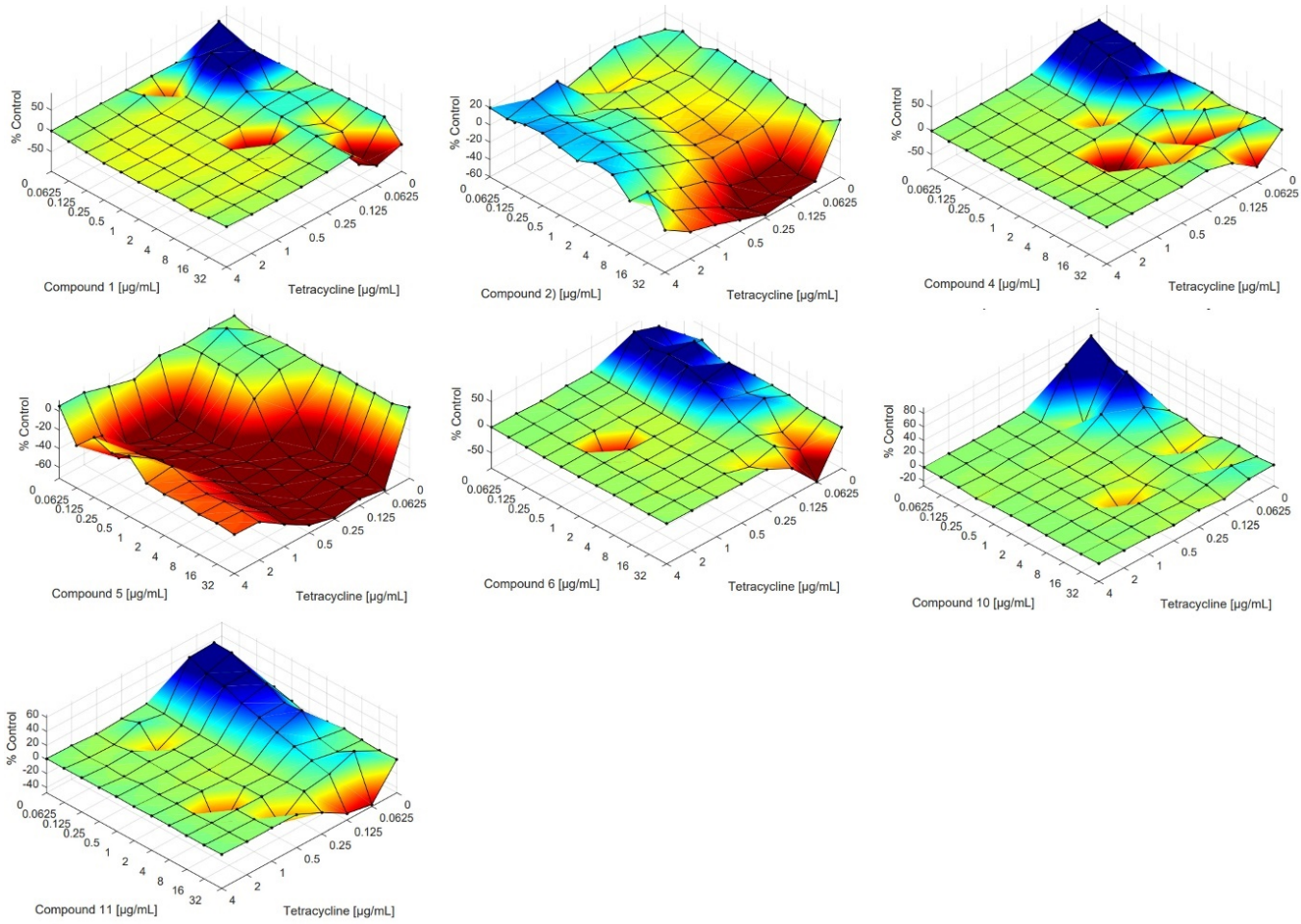

Figure 2: Synergy surface plots of the interaction between pyrazolyl sulphonamide compounds and tetracycline

Checkerboard analysis has indicated that four compounds exhibit possible synergism with the tetracycline antibiotic. We analyzed the data using a validated, open-access software tool to assess and quantify possible drug combination effects in terms of synergy versus antagonism [13, 15]. Using the classical Loewe synergy and antagonism model, we detected synergistic interaction between the compounds and tetracycline (Figure 2).

In particular, surface plots of compounds 1, 4, 10, and 11 demonstrate synergism (dark blue), especially at combinations involving concentrations below the MICs. Additionally, some minimal synergistic potential (flat) could be seen on the surface plot of the compound 6-tetracycline combination. The combination of tetracycline with compound 5 was largely antagonistic (Brown). The result of this analysis correlates with that of the checkerboard study and thus confirms potential synergism between tetracycline and the active sulphonamide derivatives.

Analysis of the potential combination interaction between the compounds and tetracycline using their respective MICs did not yield any synergistic effect (Figure 3). Antagonism was demonstrated in all combinations explored, particularly in the tetracycline antibiotic, since the impact of the combinations often resulted in weakened activity against the bacteria when compared to the effect of the test substance alone. This correlates with the findings of the surface plot analysis. Accordingly, all prospective combination analyses between tetracycline and sulphonamides should be focused on concentrations below the MICs.

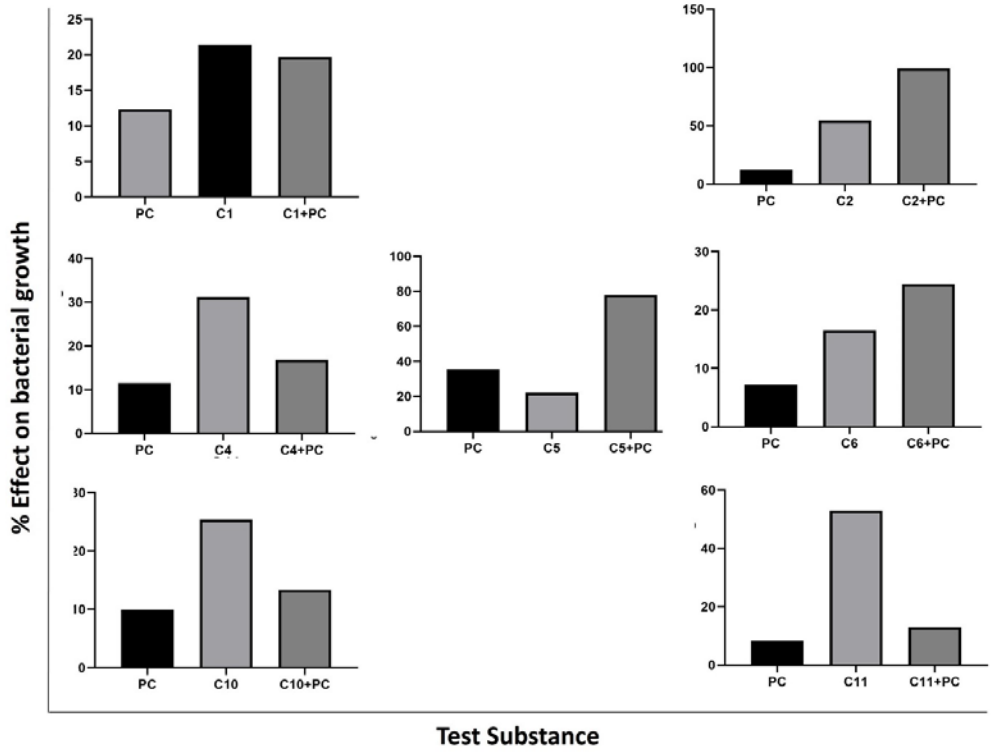

Figure 3: Interactive effects of pyrazolyl sulphonamides and tetracycline in *S. haemolyticus*. C1, Compound 1; C2, Compound 2; C4, Compound 4; C5, Compound 5; C6, Compound 6; C10, Compound 10; C11, Compound 11

CONCLUSION

The novel pyrazolyl sulphonamides investigated in this current study demonstrated potent activity against *S. haemolyticus*, with MICs of seven compounds ranging from 1 µg/mL to 16 µg/mL. This indicates the potential of the sulphonamide derivatives as a valuable resource for novel antimicrobial agents. The study also demonstrated a potential synergistic activity between sulphonamides and tetracycline. With the limited clinical usefulness of both sulphonamide antibiotics and tetracyclines because of resistance, synergistic sulphonamide derivative-tetracycline combinations could be vital for the treatment of severe drug-resistant infections caused by *S. haemolyticus* and other micro-organisms. Future research should optimize these compounds to enhance their antibacterial potency and assess their effect against other disease-causing organisms.

FINANCIAL ASSISTANCE

The Directorate of Research, Innovation and Consultancy (DRIC), University of Cape Coast, supported this study through a research support grant (RSG/INT/CoHAS/2022/101).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Ewura Seidu Yahaya, Nana Ama Amissah, Phyllis Elsie Owusu Agyei, Roland Saahene, and Martins Ekor conceived and designed the study. Jesse Azebiik Anak and Nana Ama Amissah did the data collection. Collins Obuah, Anthony Ablordey, and Michael Ainooson did an analysis and interpretation of the data. Ewura Seidu Yahaya wrote the first draft of the manuscript, and all authors read and finalized the manuscript. Funding acquisition was by Ewura Seidu Yahaya, Nana Ama Amissah, Phyllis Elsie Owusu Agyei, Roland Saahene, Anthony Ablordey, and Martins Ekor.

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