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Synergistic bactericidal profiling of prodigiosin extracted from *Serratia marcescens* in combination with antibiotics against pathogenic bacteria

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ABSTRACT

The emergence of multidrug-resistant (MDR) bacteria is on the rise and the situation has been worsening with each passing day, which is evident from the outpouring number of reports about how more and more pathogens are becoming resistant to even the third and fourth generations of antibiotics. Lately, combination therapies or drug synergy have been giving promising results in curbing infections since it delineates its action on multiple aspects as compared to monotherapies. In this study, we used prodigiosin, a bacterial pigment endowed with magnificent biological properties, in combination with six antibiotics to study its effect on *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Chromobacterium violaceum*. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of prodigiosin against the test organisms was determined and a checkerboard assay of prodigiosin with various antibiotic combinations was performed with an aim to abate antimicrobial resistance. MIC and MBC of prodigiosin was obtained in the range of 4–16 µg/mL, which was lower than that of most test antibiotics. Coupling prodigiosin with other test antibiotics exhibited an excellent synergy profile against all test organisms and the effects were reported to be either synergistic or additive. In the case of *S. aureus* and *C. violaceum*, all combinations were found to be synergic, and remarkably for *S. aureus*, FBC index was reported to be as low as ≤ 0.25 with all of the test antibiotics. Therefore, it is deduced that prodigiosin augments and intensifies the action of antibiotics, and results in a double-whammy against the MDR strains.

1. Introduction

The discovery of antibiotics has revolutionized the field of medicines and saved millions of lives over close to last two centuries [1]. However, the rapid and dramatic co-evolution of microbes against antibiotics has rusted the potency of these ‘miracle drugs’ and has ultimately rung the global alarm of antibiotic resistance. Even the World Health Organization (WHO) has confirmed this ever-increasing menace and announced the threat by stating “The world is running out of antibiotics” [2]. According to the report ‘Tackling Drug-Resistant Infections Globally’ published in May 2016, it is predicted that if proper actions are not taken by 2050, the multidrug-resistant (MDR) or extremely drug-resistant (XDR) pathogens could take lives of 10 million people every year [3]. Therefore, to address these consequences, it is imperative to devise novel approaches or develop new antibiotics or biocidal compounds at the earliest [4].

In the last couple of decades, combinational therapy has emerged as a promising strategy for treating several diseases such as cancer [5,6], diabetes [7], AIDS [8], hypertension [9], autoimmune diseases [6],

tuberculosis [10], infectious diseases [11,12] and even against the recent COVID-19 pandemic [13]. In context to drug-resistance, ‘drug cocktails’ could be a game-changer as they come with several benefits such as (1) a broadened antimicrobial spectrum, (2) the likelihood of developing drug resistance would reduce substantially as the chances of acquiring resistance towards a novel combination would be much lower, (3) it could be more efficacious against polymicrobial infections, (4) if the exercised drugs act synergistically, i.e., the combined effect of antimicrobial agents used is greater than the sum of their individual effects, it could provide even better pathogen clearance than the single drug alone and that too at a very low concentration, and (5) the lower concentration of antimicrobial agents could minimize the drug toxicity in the host [11,14].

Most clinically used antibiotics, to date, are either microbial natural metabolites or semisynthetic derivatives of these molecules [15]. However, the conventional antibiotics are lately failing in their efficacy towards the MDR pathogens. Therefore, scientists and pharmaceutical companies are diving deep into the bacterial sources, particularly searching for novel potent bacteria to discover a unique backbone of

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antibiotics that can outperform in the race between pathogens and drug discovery. Amongst many such candidates, a crimson red bacterial pigment called Prodigiosin, is one such alkaloid secondary metabolite produced by numerous microorganisms including *Serratia marcescens*, *S. rubidaea*, *Pseudomonas magnesorubra*, *Pseudovibrio denitrificans*, *Pseudoalteromonas rubra*, *Vibrio psychroerythrosus*, *V. gazogenes*, *Streptomyces lividans*, *Nocardia* spp., etc. [16]. It contains three pyrrole rings in its molecular structure. True to its name, this natural pigment is prodigious and manifests multifaceted characteristics such as antimicrobial, anticancer, antimalarial, antifungal, immunosuppressive, etc. [16,17]. These phenomenal potentials of prodigiosin have caught the attention of many researchers, nutraceutical, pharmaceutical, cosmetic and other industries [18].

The aim of this study was to evaluate the action of prodigiosin as an antimicrobial agent against infectious microorganisms. Most importantly, we investigated the synergistic efficacy of prodigiosin with antibiotics, the amalgamation of which could intensify the potency of conventional antibiotics against the pathogenic microorganisms as a novel resolution that could be used to address drug-resistance issues.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Microorganisms used in experiments were *P. aeruginosa* MG, *P. aeruginosa* PG30, *Staphylococcus aureus*, *Chromobacterium violaceum* and *S. marcescens*. The strains *P. aeruginosa* MG, *S. aureus*, *C. violaceum* and *S. marcescens* were gifted by Institute of Science, Nirma University, Ahmedabad, India. *P. aeruginosa* PG30 was isolated from wastewater sample from Pirana landfill, Ahmedabad, India. Both of the *P. aeruginosa* strains were routinely cultured overnight in *Pseudomonas* broth [peptone 30 g/L (HiMedia, India), potassium sulfate 10 g/L (Merck, Germany), magnesium sulfate 1.4 g/L (Merck, Germany), glycerol 3% (HiMedia, India)] at 37 °C at the agitation speed of 200 rpm. All the other organisms were cultured in Luria-Bertani broth (HiMedia, India) overnight at 37 °C (except for *S. marcescens*, which was grown at 28 °C) at the agitation speed of 200 rpm, and their purity was ascertained by streaking on agar plates the day after. *S. marcescens* was used for the extraction of prodigiosin while all others were used as test microorganisms. Glycerol stocks of the strains were maintained and preserved in 25% glycerol at -80 °C for further use.

2.2. Extraction of prodigiosin pigment from *S. marcescens*

Overnight grown activated culture of *S. marcescens* was spread on LB agar (HiMedia, India) and incubated at 28 °C for 96 h. The cells were scraped, washed twice with sterile distilled water and collected in 20 mL acidified methanol (4 mL of 1 N HCl in 96 mL of methanol) and the suspension was vortexed for 5 min. This was followed by centrifugation (Eppendorf 5810 R, Germany) at the agitation speed of 7500 rpm for 15 min at 4 °C. The methanol extract was left to evaporate to dryness at 28 °C, following which the residue was re-suspended in 1 mL acidified methanol and stored between 0–4 °C for further use [19].

2.3. Quantification and characterization of prodigiosin

The concentration of prodigiosin was determined by the standard curve equation based on absorbance at 492 nm [20]. The extracted prodigiosin was characterized through ultraviolet-visible spectroscopy (Shimadzu UV-1900, Japan) and a spectrum was generated in the range of 300–700 nm. The purity of prodigiosin was determined by HPLC (Shimadzu LC-20AT, Japan) using Kinetex C18 column (250 × 4.6 mm, 5 µm, Phenomenex, USA). The separation was achieved using methanol and water (HPLC grade, Merck, Germany) as the mobile phase at a flow rate of 1 mL/min.

2.4. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC of prodigiosin and the antibiotics used in this study were determined through microbroth dilution assay in 96-well flat-bottom sterile microplates (SPL Life Sciences, Korea) [17,21]. 100 µL of 2X Mueller-Hinton (MH) broth (HiMedia, India), 10% v/v (20 µL) standardized microbial inoculum and 80 µL of varying concentrations of prodigiosin (1–128 µg/mL) or antibiotics (1–2048 µg/mL) were added into each well. Controls such as sterility control and growth control were also considered for observation. The desired microbial inoculum was prepared using overnight grown culture which was standardized as per 0.5 McFarland turbidity standard at an optical density of 600 nm (OD₆₀₀) (Systronics Visiscan Spectrophotometer 167, India) and adjusted with sterile normal saline solution (0.85% NaCl). Antibiotics used in the experiments were ampicillin (stock solution - 100 mg/mL), chloramphenicol (34 mg/mL), spectinomycin (50 mg/mL), tetracycline (10 mg/mL), kanamycin (50 mg/mL) and nalidixic acid (10 mg/mL). All the antibiotics were purchased from HiMedia, India. The microtiter plates were incubated at 37 °C for 24 h. The lowest concentration that inhibited the visible growth of the organism was considered to be the MIC, while the minimum concentration at which no viable cells were found after 24 h incubation at 37 °C when streaked on an MH agar plate in the absence of antibiotic was recorded as the MBC. All experiments were performed in triplicates.

2.5. Checkerboard assay

Synergistic antimicrobial profiling of prodigiosin with different antibiotics against the pathogenic bacterial strains was constructed using one of the authenticated procedure used for determining the synergistic effect as suggested by American Society for Microbiology (ASM): the checkerboard titration assay [22,23]. In this study, different concentrations of prodigiosin and antibiotics were combined in a 2-dimensional fashion with the test organisms in 96-well flat-bottom sterile microtiter plates. Thus, each well contained a unique combination of prodigiosin and antibiotic concentration against test pathogens. The inoculum for the test organisms was standardized as described earlier. Controls were set as stated earlier. The plates were incubated at 37 °C for 24 h and MBC was determined. For assessment of results, fractional bactericidal concentration (FBC) index [24,25] was determined by calculating the sum of FBC of prodigiosin and the antibiotic (i.e., $\sum \text{FBC index} = \text{FBC}_{\text{prodigiosin}} + \text{FBC}_{\text{antibiotic}}$). FBC can be defined as the MBC of drug used in combination divided by the MBC of the drug when used alone. The interaction is considered as synergistic when the FBC index is found to be ≤ 0.5 ; additive when the FBC index is > 0.5 to ≤ 1.0 ; indifferent when the FBC index is > 1.0 to ≤ 2.0 and antagonistic when FBC index is > 2.0 [22].

$$\text{FBCI} = \frac{\text{MBC of PRO in combination with ATB}}{\text{MBC of PRO alone} + \frac{\text{MBC of ATB in combination with PRO}}{\text{MBC of ATB alone}}}$$

where, FBCI = FBC index, MBC = minimum bactericidal concentration, PRO = prodigiosin, ATB = antibiotic.

3. Results

3.1. Characterization of the prodigiosin

As per Darshan and Manonmani [19], prodigiosin gives absorption spectrum maxima at 535 nm. A single peak at 535 nm was obtained in the UV-visible absorption spectrum of prodigiosin confirming its purity (Fig. 1). The purity of the prodigiosin extracted from *S. marcescens* was up to 95% as determined by HPLC. The HPLC profile of prodigiosin

pigment is shown in Fig. 2.

3.2. Prodigiosin antimicrobial activity

The MIC as well as MBC of prodigiosin and antibiotics against test pathogens are given in Table 1. Prodigiosin showed excellent bactericidal activity against all tested pathogenic strains. In all assays, MIC and MBC of prodigiosin ranged from 4 to 16 µg/mL. *P. aeruginosa* MG and *C. violaceum* were found to be resistant to ampicillin. In majority (75%, $n = 18$ out of 24 cases; Note: two resistant cases were also included in total cases) of the cases, prodigiosin alone as an antimicrobial agent exhibited much lower MBC as compared to the test antibiotics. Apart from that, in a few cases (37.50%, $n = 9/24$), the MBC of prodigiosin was even ≤ 128 -fold lower than the MBC of test antibiotics. In a way, it could also be claimed that against all the test organisms, prodigiosin was found to be more or equally effective than ampicillin, chloramphenicol, spectinomycin and nalidixic acid as the obtained MBC of prodigiosin was lower than or equal to the said antibiotics. Interestingly, in both *P. aeruginosa* strains, the MBC concerning prodigiosin was lower than all the tested antibiotics.

3.3. Synergistic profiling of prodigiosin with antibiotics

The combination effect of prodigiosin with commercially available test antibiotics against pathogenic microorganisms is given in Table 2 and depicted in Fig. 3. This bacterial pigment manifested an outstanding synergistic effect with antibiotics against test organisms. In all combinations, the effect was either synergistic (63.64%, $n = 14/22$) or additive (36.36%, $n = 8/22$). No indifferent or antagonist type of activity was observed in any combination. Since *P. aeruginosa* MG and *C. violaceum* were found to be resistant against ampicillin, the checkerboard assay for the same was left unattempted as concocting a combination was impossible. For *S. aureus*, prodigiosin displayed a remarkable synergistic effect in all combinations with an FBC index of ≤ 0.25 , which is half of the required value to be synergic (i.e., 0.50). Similarly, the combinations were also found to be synergistic against *C. violaceum* in all attempted assays. For *C. violaceum*, chloramphenicol and nalidixic acid were noted to be the most efficacious with prodigiosin, giving an FBC index as low as 0.19. In the case of *P. aeruginosa* MG, the effect was synergistic with chloramphenicol and nalidixic acid, while additive with spectinomycin, tetracycline, and kanamycin. The combination results were additive with every single exercised antibiotic for *P. aeruginosa* PG30, except for nalidixic acid (synergistic with FBC index = 0.50). Mean FBC index for all assays was found to be 0.46, whereas, for *S. aureus* case, it was calculated to be as low as 0.165.

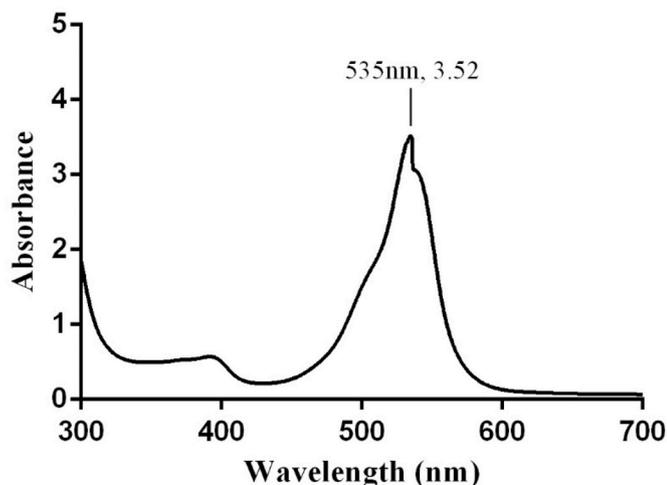


Fig. 1. UV-vis absorption spectrum of prodigiosin pigment.

4. Discussion

A serious shortfall of new antibiotics has dragged the world towards global health emergencies [2,26]. In response to defeat this growing threat, a pressing need has arisen to discover novel biocidal substances or to find an alternative approach to eradicate MDR issues [4]. Combination antimicrobial therapy (CAT) is a potent approach that can be used to treat many infectious diseases [12]. Many studies have shown that CAT has an edge over the monotherapy of an antibiotic against infections [11,12,21,22]. In our attempt to prove so, prodigiosin, an exemplary bacterial pigment known for its innumerable properties was used. The amphiphilic nature of prodigiosin endows it to easily penetrate through the bacterial cell membrane and affect its integrity [27, 28]. Thereafter, it inhibits the action of DNA gyrase and topoisomerase, the enzymes that are obligatory for DNA replication [16,29].

It is proven that microorganisms communicate with each other via exchange of signalling molecules or auto-inducers through a phenomenon commonly known as quorum-sensing that plays a pivotal role in expressing pathogenicity, motility, biofilm formation, production of antibiotics and pigments, spore formation and many others [26]. In clinical infections, pathogenic microorganisms cause threats majorly by forming a biofilm. By doing so, the capability of delineating resistance towards antibiotics in pathogens could increase up to 1000–1500 times [30]. Amongst the biofilm producers, *P. aeruginosa* is renowned for its ability to produce very strong biofilm, which has made this superbug to be recognized as a ‘critical priority’ pathogen in the list of “antibiotic-resistant priority pathogens” published by WHO [31]. In the present study, *P. aeruginosa* (as well as *C. violaceum*) showed resistance to ampicillin, a β -lactam antibiotic that is a potent cell wall synthesis inhibitor. According to the data shown by Darshan and Manonmani [19], the obtained MBC for *P. aeruginosa*, *Escherichia coli*, *S. aureus* and *Bacillus cereus* were 16, 20, 9 and 12 µg/ml, respectively. These results are in agreement with the data sets obtained in the present study. In synergistic profiling of both *P. aeruginosa*, additive effect was obtained with most antibiotics which is predicted to be due to the formation of strong biofilm by these strains that might have hindered the penetrating ability of antimicrobials to enter the cell [21,32].

Similar to *P. aeruginosa*, *S. aureus* is also amongst the ‘high priority’ pathogens [31] and falls under the class of ESKAPE pathogens (six pathogens exhibiting MDR and virulence: *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa* and *Enterobacter* spp.) [33]. It is also regarded as the most common human pathogen that causes a wide range of infections. In an extensive study of prodigiosin against twenty oxacillin-resistant clinical isolates of *S. aureus*, the MBC was manifested to range from 2 to 16 µg/ml [17]. These results are also consistent with the present study, where the MIC and MBC value against *S. aureus* was 8 and 16 µg/ml, respectively. Likewise, Suryavanshi et al. [34] have also reported extraordinary MIC value (2.5 µg/ml) against *S. aureus*. Notably, combinations of prodigiosin with all tested antibiotics against *S. aureus* gave an excellent FBC index of ≤ 0.25 , which was half of the required value to be synergic (i.e., 0.50). In other words, *S. aureus* got even more sensitized to prodigiosin in the presence of antibiotics. Numerous studies have reported that gram-positive bacteria, especially *S. aureus*, are more susceptible to prodigiosin compared to gram-negative bacteria [16–18,34]. Our synergistic profiling results of *S. aureus* are concurrent with the above-mentioned studies.

Apart from that, opportunistic pathogen *C. violaceum* was also considered in our study, even though the infections caused by this microbe are rare, though are usually fatal [35]. The combined effect of prodigiosin with antibiotics was synergistic, suggesting that prodigiosin can potentiate the action of commercially available antibiotics against this pathogen. We predict that prodigiosin orchestrates the membrane integrity of the bacterial cell and promotes the entry of itself and other antibiotics into the cell by altering the membrane permeability.

Recently, prodigiosin was also tested for a synergistic effect against

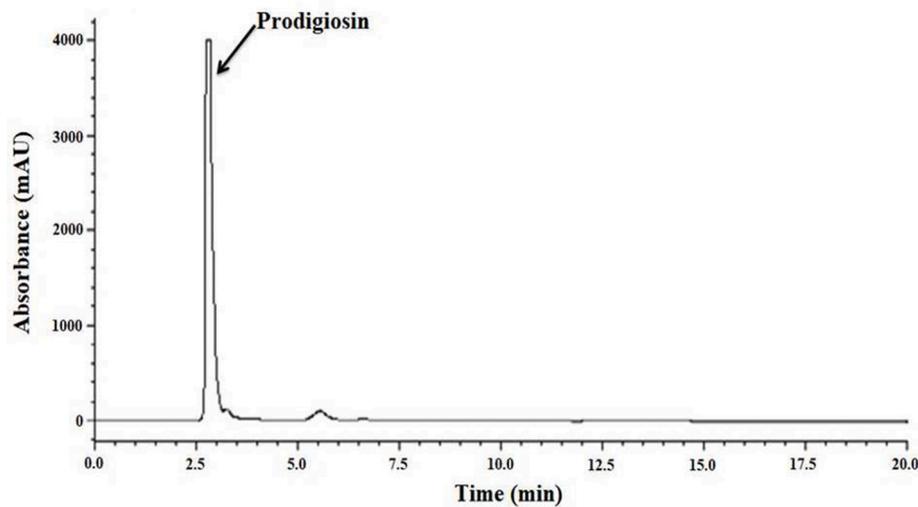


Fig. 2. HPLC elugram of prodigiosin pigment. The prodigiosin peak is marked with a black arrow. The purity of prodigiosin is 95% as determined by HPLC.

Table 1
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of prodigiosin and antibiotics against different pathogens.

Agents (µg/ml)	<i>Pseudomonas aeruginosa</i> MG		<i>Pseudomonas aeruginosa</i> PG30		<i>Staphylococcus aureus</i>		<i>Chromobacterium violaceum</i>	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
PRO	16	16	4	4	8	16	16	16
AMP	R	R	1024	2048	1024	2048	R	R
CHL	256	256	4	8	16	32	8	16
SPC	1024	2048	512	1024	1024	2048	8	16
TET	16	32	32	64	4	8	1	2
KAN	16	32	512	512	8	8	16	16
NAL	512	1024	1024	1024	64	128	16	32

PRO: prodigiosin; AMP: ampicillin; CHL: chloramphenicol; SPC: spectinomycin; TET: tetracyclin; KAN: kanamycin; NAL: nalidixic acid; R: resistant.

Table 2
Synergistic antimicrobial interaction of prodigiosin with antibiotics of clinical use.

Agents (µg/ml)	<i>Pseudomonas aeruginosa</i> MG				<i>Pseudomonas aeruginosa</i> PG30				<i>Staphylococcus aureus</i>				<i>Chromobacterium violaceum</i>			
	PRO*	ATB [†]	FBC [‡]	RES [§]	PRO*	ATB [†]	FBC [‡]	RES [§]	PRO*	ATB [†]	FBC [‡]	RES [§]	PRO*	ATB [†]	FBC [‡]	RES [§]
AMP	-	R	-	-	3	128	0.81	A	1	128	0.12	S	-	R	-	-
CHL	4	48	0.44	S	2	2	0.75	A	1	6	0.25	S	2	1	0.19	S
SPC	7	256	0.56	A	3	16	0.76	A	1	128	0.12	S	7	1	0.50	S
TET	7	14	0.87	A	1.5	16	0.62	A	1	0.5	0.12	S	4	0.5	0.50	S
KAN	10	6	0.81	A	3	98	0.94	A	1	1	0.19	S	4	1	0.31	S
NAL	7	16	0.45	S	1	256	0.50	S	2	8	0.19	S	2	2	0.19	S

PRO*: minimum bactericidal concentration (MBC) of prodigiosin in association with antibiotic; ATB[†]: MBC of antibiotic in association with prodigiosin; AMP: ampicillin; CHL: chloramphenicol; SPC: spectinomycin; TET: tetracyclin; KAN: kanamycin; NAL: nalidixic acid; FBC: fractional bactericidal concentration index; R: resistance; RES: result; S: synergistic; A: additive; PRO: prodigiosin; ATB: antibiotic.

[‡] FBC index = (MBC of PRO in association with ATB/MBC of PRO alone) + (MBC of ATB in association with PRO/MBC of ATB alone).

[§] FBC index ≤0.5: synergy; FBC index >0.5 to ≤1.0: additive.

Mycobacterium smegmatis with 9 lesser preferred cell wall synthesis inhibiting antibiotics [25]. Prodigiosin presented a considerable MIC value of 2.8 against *M. smegmatis*, which was lower than all of the used antibiotics, except for vancomycin. In the study, prodigiosin was found to be either synergistic (66.67%, n = 6/9) or indifferent (33.33%, n = 3/9), thus suggesting prodigiosin as a promising candidate to be used as a supplement to potentiate the cell wall synthesis inhibitors. Besides antibiotics, the synergism of prodigiosin with biosurfactants [36,37] and with chitinolytic enzymes [38] has already been reported for antibacterial and antifungal activities, respectively. From these studies, it was deduced that prodigiosin intensifies the efficacy of the amalgamated agents against pathogenic microorganisms.

Similar to prodigiosin, a purple-hued bacterial pigment violacein produced by *C. violaceum*, *Janthinobacterium lividum*, *Alteromonas*

luteoviolacea, *Duganella* sp. etc. has also exhibited tremendous synergistic antimicrobial efficiency against *S. epidermidis* [21], *Salmonella typhi*, *Vibrio cholerae*, *P. aeruginosa*, *K. pneumoniae* and *S. aureus* [22] and connoted its potential to be used in combination with antibiotics.

5. Conclusion

The world is experiencing a serious shortfall in its available antibiotic arsenal. With the emergence of some highly resistant superbugs in tandem with very few novel antibiotics in the pipeline, it is time to look for alternative approaches besides developing next-generation antibiotics. Combining the proficiency of novel molecules such as prodigiosin with commercial antibiotics may prove to be a plausible substitute to subjugate the surging morbidity and mortality. Prodigiosin can be a

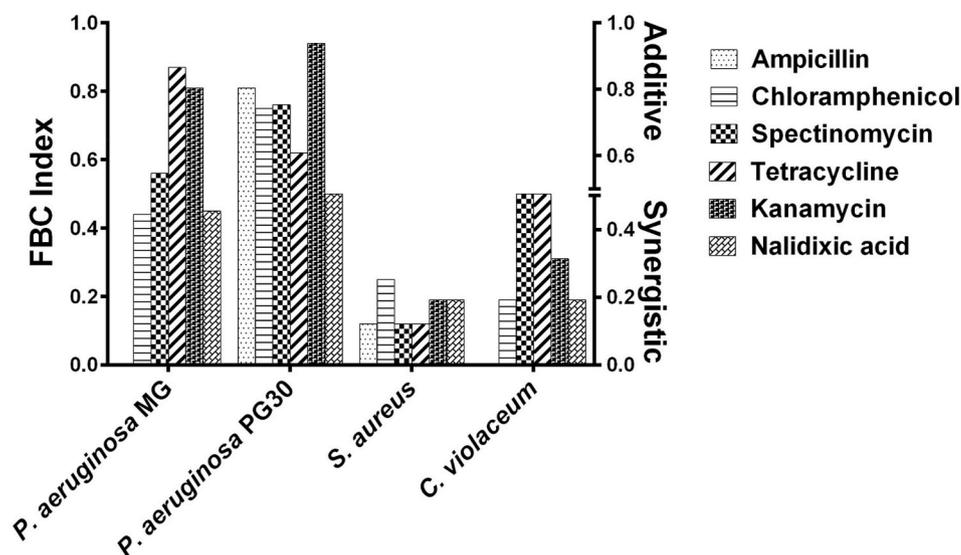


Fig. 3. Fractional bactericidal concentration (FBC) index of prodigiosin with commercial antibiotics against pathogenic bacteria. Prodigiosin in combination with test antibiotics showed synergism against most test pathogens. In certain combinations, additive effect was reported against both *P. aeruginosa* strains. Antagonist or indifferent type of activity was not obtained at all. Since both *P. aeruginosa* and *C. violaceum* were found to be resistant against ampicillin, the checkerboard board assay for the same was not performed. FBC index is signified by ≤ 0.5 : synergy; >0.5 to ≤ 1.0 : additive; >1.0 to ≤ 2.0 : indifferent and >2.0 : antagonism.

treasured chemical of inestimable value owing to the range of function it is involved in, naming from antimicrobial, anti-tumorigenic, antimalarial, immunosuppressive and even as a food colourant. Though it is predicted to have different mechanisms that it uses to execute its aforementioned functions on different hosts, the exact molecular target still remains an enigma. With regard to its antibacterial properties, it is proposed to function as a bacteriolytic and bacteriostatic agent during the exponential and stationary phases of the growth cycle, respectively [39]. In our study, it is evident that in most cases, prodigiosin when coupled with antibiotics having different modes of action was able to display synergism and wipe out pathogens at minimal possible concentrations. In some cases, these combinations depicted additive responses but in absolutely no case did we get antagonistic or insignificant interaction. Therefore, in a nutshell, fusing prodigiosin with antibiotics may increase the efficacy of the drugs by several folds, which in the future, might lead to its emergence as a compelling substitute providing the same effects as antibiotics alone, that too at a much lower concentration.

Author Statement

Nisarg Gohil: Conceptualization, Methodology, Investigation, Data curation, Writing - original draft, Visualization; Gargi Bhattacharjee: Investigation, Writing - original draft, Writing - review & editing; Vijai Singh: Conceptualization, Supervision, Validation, Writing - review & editing.

Declaration of competing interest

All authors have declared the no conflict of interest.

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