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Study on Antimicrobial Potential of Selected Non-antibiotics and its Interaction with Conventional Antibiotics

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Abstract

The escalating levels of antimicrobial drug resistance render it indispensable to explore newer drugs with lesser degrees of toxicity and with fewer chances of developing resistance. Various studies on the discovery of novel antimicrobials have found different degrees of antimicrobial activity in commonly used medicines with diverse pharmacological actions i.e., non-antibiotics. The present work aimed to describe qualitatively and quantitatively *in vitro* antimicrobial activity of selected non-antibiotic drugs i.e., Acetyl salicylic acid, Methyldopa, Propranolol and Fluoxetine alone and in combination with three conventional antimicrobial drugs i.e., Ciprofloxacin, Benzyl penicillin, and Fluconazole against three standard test microorganisms, i.e., *E. coli, S. aureus* and *C. albicans.* Agar well diffusion method was used for testing antimicrobial sensitivity, while the drug interaction was estimated using fractional inhibitory concentration index (FIC index) obtained from checkerboard broth dilution method. All the four non-antibiotics tested for antimicrobial activity showed activity against at least one tested microorganism, whereas fluoxetine showed antimicrobial activity against all tested organisms. Combined effect of fluconazole + fluoxetine and fluconazole + propranolol against *C. albicans* showed *synergistic* activity based on the FIC_{index} value obtained i.e., 0.25 and 0.1875, respectively. Based on the results, study suggests that fluoxetine among the other non-antibiotics has a potential for being developed into an effective antimicrobial agent. However, the study needs to be extended in the future to determine the in vivo antimicrobial activity.

1 Introduction

Antibiotics forever changed the way we treat infectious diseases and are considered as one of our significant arms in fighting microbial infections. However, over the past few decades, the use of antibiotics is becoming increasingly restricted despite the fact that they exist in large numbers[1]. The reason behind such a rapid decline in the market of antibiotics is largely attributed to the emergence of drug resistant microbes, which render some of the broadest spectrum antibiotics ineffective[2]. Moreover, the toxic side effects produced by some antibiotics also reduce their market demand. Anti-microbial drug resistance is a serious global health issue compromising the treatment of various infections, i.e., minor and life-threatening[3]. Thus, the escalating levels of drug resistance render it indispensable to explore newer drugs with lesser degrees of toxicity and possibly fewer chances of developing resistance[4].

New drug discovery or new drug combinations may be a potential solution to combat resistance development in serious infectious diseases and at the same time priority should also be given to novel treatment methods before antimicrobial resistance robs us of our current antibiotics. The concept of reversal of resistance by means of non-antibiotics could be a promising solution for bringing back drug resistant micro-organisms to their original sensitivity to the classical antibiotics.

Antihistamines[5-7] tranquilizers[8], antihypertensives[9], antipsychotics[10-14] and anti-inflammatory agents[15-16] are examples of some classes of medicine that exihibited limited to significant antimicrobial action.

Such compounds, having antimicrobial properties in addition to their pre-designated pharmacological actions, have been considered as 'Non-antibiotics'. Ehrlich also noticed the similar potential for antimicrobial action in psychoactive drugs while developing antimicrobial from dyes[17-18].

Moreover, some of these non-antibiotic agents have been found to demonstrate synergism when combined with conventional antimicrobials. This two-fold advantage of non-antibiotics i.e. their antimicrobial activity and synergy with antibiotics could help in delaying the emergence of antimicrobial resistance. For example, Kristiansen *et al* found that nizatidine and omeprazole augmented the antibacterial action of metronidazole against *Helicobacter pylori*[19]. Synergistic effect between non-antibiotic compounds and antibiotics enables the use of the respective antibiotics when their effectiveness as single agents is reduced[20]. Such studies open up the possibilities to treat problematic infections such as those of multi drug resistant (MDR) phenotypes.

Based on our literature review, there were no definitive studies that demonstrated the antimicrobial activity of acetyl salicylic acid, methyldopa, propranolol and fluoxetine and its combined effect with antibiotics. Hence, present study aimed to determine the *in vitro* antimicrobial potential of above mentioned non-antibiotics that are available in Eritrea against standard test microorganisms, i.e., *E. coli, S. aureus* and *C. albicans* and their interaction with conventional antimicrobial drugs such as ciprofloxacin, benzyl penicillin, and fluconazole against respective microorganisms qualitatively and quantitatively using FIC index (∑FIC) and Checkerboard assay.

2 Materials and Methods

2.1 Chemicals used

Ciprofloxacin, Benzyl penicillin, Fluconazole, Acetyl salicylic acid, and Propranolol as a pure pharmaceutical grade dry powder form were obtained from Azel Pharmaceuticals Sh. Co, Keren, Eritrea as a gift samples and Fluoxetine 20mg was obtained from St. Merry Psychiatric Hospital, Asmara, Eritrea. Mueller Hinton Agar Media (Himedia Laboratory PLC, Mumbai, India) and Nutrient Broth (Himedia Laboratory PLC, Mumbai, India) were used for microbiological studies. All the other chemicals used were of analytical grade and obtained locally.

2.2 Test microorganisms

A total of 3 stock microorganisms obtained from the Eritrean National Health Laboratory (ENHL) was used for the antimicrobial assay. The test microorganisms consists of a Gram positive bacteria i.e., *Staphylococcus aureus* (ATCC 25923), a Gram negative bacteria i.e., *Escherichia coli* (ATCC 25922) and a fungi (Yeast) i.e., *Candida albicans* (ATCC 10231).

2.3 Inoculum preparation and standardization

All the stock organisms were inoculated on Muller Hinton Agar and incubated for 24 – 48 h at 37^{o}C. The inoculum for antimicrobial sensitivity test was prepared from overnight culture of respective test microorganisms. These colonies were then mixed with sterile normal saline and diluted till the turbidity was visually comparable to 0.5 McFarland turbidity standard, which is equivalent to a bacterial count of approximately 1 X 10^6 CFU/ml[21].

2.4 Drug sample preparation

All the drug sample solutions used in this study were freshly prepared in distilled water at a concentration of 1 mg/ml for antibiotics and 2 mg/ml for all the non-antibiotics.

2.5 Testing for antimicrobial activity

2.5.1 Antimicrobial sensitivity test of non-antibiotics and antibiotics

Antimicrobial activity of non-antibiotics i.e., aspirin, methyldopa, propranolol and fluoxetine was tested by agar well diffusion method using Muller Hinton agar medium. All the overnight culture (Turbidity adjusted to 0.5 McFarland standard) of test microbes were inoculated into Muller Hinton agar plates using sterile cotton swab. While inoculation, the plate was rotated 90^{o} each time to ensure an even distribution of inoculum and then about 5 mm well was made using sterile borer. The wells made were having 10mm away from the plate wall and 15mm from each well to minimize overlap of inhibition and confusion. Then 50 µl of each drug sample was added into each well and incubated at 37°C for 24 hours for bacterial pathogens and at 30°C for 48 hours for fungal pathogen. The antimicrobial activities were assessed by the presence or absence of inhibition zones and by measuring the diameter of the zone of inhibition formed around the disks. Antimicrobial activity of antibacterial antibiotics i.e., ciprofloxacin, benzyl penicillin and antifungal antibiotic i.e., fluconazole was tested using the same procedure as mentioned above for non-antibiotics[22].

2.5.2 Antimicrobial sensitivity test of non-antibiotics and antimicrobials in combination[23]

Combined antimicrobial activity of antibiotics and non-antibiotics was performed using above mentioned method. The concentration of each drug tested in combination was same as the concentrations used in antimicrobial sensitivity testing of both antimicrobials and non-antibiotics alone. Based on the definition of additivity and synergism by Johnson *et al*[24] interaction of drug combinations will be determined and the results obtained will be used as a baseline information for quantifying the activity by using checkerboard assay.

2.5.3 Determination of MIC

Based on the zone of inhibition measurement, MIC value of propranolol and fluoxetine was determined using nutrient broth media, since they showed superior synergistic activity. Different drug concentrations range were prepared in test tubes by double dilution method i.e., 2000 - 3.90.5 µg/ml for fluoxetine and 10 - 0.0195mg/ml for propranolol.

Each 100µl of different drug dilutions were transferred to separate test tubes containing 5ml of nutrient broth and 20µl of standardized microbial suspension. Then the mixture was incubated at 37^0C for 48 h. The lowest concentration of drug in a tube that failed to show any visible or macroscopic growth or turbidity after gently vortexing was considered as MIC of that particular drug. MIC value of the drugs will form the basis for the determination of FIC_{index} using checkerboard technique. The MIC determination was performed in duplicate for each organism, and the experiment was repeated where necessary[25].

2.5.4 FIC Index determination by checkerboard technique

According to the satisfactory result of synergy found in antimicrobial activity of combined antimicrobial and non-antibiotics, FIC_{index} of the two drug combinations namely fluconazole + propranolol and fluconazole + fluoxetine was determined against *C. albicans* using checkerboard assay to confirm quantitative degree of synergy.

The activity of drugs in combination was investigated using the checkerboard broth dilution method. Two fold serial dilutions of the antifungals and non-antibiotics were prepared for each combinations tested and 100 µl aliquots of each component was placed into the wells of the sterile test tubes. The inoculum was prepared using the same method as described above in MIC determination. The test tubes were then incubated at 35^0C and MIC was determined after 24 h of incubation. Then the FIC_{index} value of the two test combinations was calculated based on the mass-action law principle by Chou and Talalay i.e., the fractional inhibitory concentration index (∑FIC) was calculated as ∑FIC = FIC A + FIC B where

FIC_A = MIC of drug A tested in combination / MIC of drug A tested alone

FIC_B = MIC of drug B tested in combination / MIC of drug B tested alone

Finally the FIC index value of the two test combinations would be interpreted as synergistic if ∑FIC ≤ 0.5, additive if ∑FIC > 0.5 and ≤ 4, antagonistic if ∑FIC > 4[26].

3 Results

The results of zone of inhibition for aspirin, propranolol, methyldopa and fluoxetine are shown in table 1. Neither antimicrobial control nor growth control showed any contamination or growth indicating controlled working environment for all the tests done. From the results, it was found that all the four non-antibiotics tested for antimicrobial activity showed activity against at least one tested microorganism, i.e., *E. coli* at a concentration of 2mg/ml, whereas fluoxetine showed antimicrobial activity against all tested organisms.

3.1 Antimicrobial activity of antibiotics + non-antibiotics combination

The results of antimicrobial activity of non-antibiotics and antibiotics in combination are shown in table 2. In the qualitative interpretation of interactions among the combination, we considered the result as a mean with *95% confidence interval*. Out of the twenty combinations used, three combinations (15%) showed synergism, twelve combinations (60%) showed additive while five combinations (25%) showed antagonistic effects, based on the definition of additivity and synergism by Johnson *et al*. The combinations fluconazole + fluoxetine and fluconazole + propranolol showed synergism against *C. albicans*. Similarly, benzyl penicillin + propranolol showed synergy against *E. coli*. Whereas, fluconazole + aspirin, fluconazole + methyldopa against *C. albicans*, benzyl penicillin + fluoxetine and benzyl penicillin + aspirin against *E. coli* and ciprofloxacin + aspirin against *S. aureus* showed antagonistic drug interaction.

Percentage of an increase in a zone of inhibition, after the drug was used in combination for benzyl penicillin + propranolol and penicillin + fluoxetine was by 19% and 98%, respectively. Similarly, when fluconazole was combined with propranolol and fluoxetine, percentage of an increase in surface area of zone of inhibition was by 132.7% and 161.6%.

3.2 MIC of propranolol, fluoxetine and fluconazole

The MIC value of non-antibiotics fluoxetine, propranolol and fluconazole against *C. albicans* was found to be 62.5 µg/ml, 5 mg/ml and 12.5 µg/ml, respectively.

3.3 FIC index by checkerboard technique

The interaction of the non-antibiotics with antibiotics against test microbial species was determined by the checkerboard method. The MIC of fluconazole alone was found to be 12.5 µg/ml. When combined with propranolol and fluoxetine, the MIC value of fluconazole reduced to 1.5625 µg/mL and 0.78125 µg/mL, respectively. When tested against *C. albicans*, fluoxetine showed synergism with fluconazole. Propranolol showed synergism with Benzyl penicillin against *E. coli* and with fluconazole against *C. albicans*. The antifungal activity of fluconazole + propranolol and fluconazole + fluoxetine combinations are shown in checkerboard tables 3 and 4, respectively. The FIC_{index} value obtained for both propranolol + fluconazole and fluoxetine + fluconazole drug combination was found to be 0.25 and 0.1875 respectively (Table 5). Thus, the

results of above two drug combinations were found to be synergistic.

Table 1: Antimicrobial activity of antibiotics and non-antibiotics alone

Test Microorganism	Zone of inhibition (mm)						
	Antibiotics			Non-antibiotics			
	CIPR	BPEN	FLUC	ASA	MDOP	PROP	FLUX
E. coli	40	22	-	16	20	13	34
S. aureus	38	23	-	R	R	R	20
C. albicans	-	-	13	R	R	R	12

CIPR = ciprofloxacin, BPEN = benzypenicilln, FLUC = fluconazole, ASA = aspirin, MDOP = methyldopa, PROP = propranolol, FLUX = fluoxetine, R = Resistance

Table 2: Combined antimicrobial activity of antibiotics and non-antibiotics

Drugs	*E. coli*		*S. aureus*		*C. albicans*
	CIPR	BPEN	CIPR	BPEN	FLUC
ASA	35 (A)	17 (AN)	31 (AN)	18 (A)	R (AN)
MDOP	36 (A)	22 (A)	35 (A)	21 (A)	R (AN)
PROP	38 (A)	24 (S)	37 (A)	19 (A)	21 (S)
FLUX	37 (A)	31 (AN)	37 (A)	21 (A)	26 (S)

A= additive, AN= antagonistic, S= synergistic

Table 3: Combined antifungal property fluconazole + propranolol

Concentration of PROP (mg/ml)	Concentration of FLUC (µg/ml)								
	0	0.78125	1.5625	3.125	6.25	12.5	25	50	100
0	+	+	+	+	+	+	-	-	-
0.625	+	+	+	+	+	+	-	-	-
1.25	+	+	+	+	+	-	-	-	-
2.5	+	+	+	+	-	-	-	-	-
5	+	+	+	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-

+ Indicates presence of microbial growth, - indicates absence of microbial growth

4 Discussions

Based on the results obtained, it was observed that *E. coli* which is naturally resistant to many conventional antimicrobial drugs showed the highest degree of susceptibility to all the non-antibiotics. Whereas, *C. albicans* and *S. aureus* was found to be susceptible only to fluoxetine.

The order of antimicrobial activity for non-antibiotics against *E. coli* can be represented as Fluoxetine> Methlydopa> Aspirin> Propranolol. The precise mechanism by which these non-antibiotics exert their antimicrobial effects is not yet known. However, the possible mechanism for their effects could be due to inhibition of cell wall formation, cell membrane distraction or inhibition of cell division. But it needs further detailed investigation to clearly state their mechanism for antimicrobial effects.

Our results on a combined effect of non-antibiotics and antibiotics is comparable with Borisy *et al.,* statement that synergistic drug pairs are rare to find, which was about 4-10% on his work on reviewing antimicrobial activity of non-antibiotics[27].

The results of percentage area of increase in inhibition zone was comparable with findings of Debnath *et al*., on his experimental evaluation of synergistic action between streptomycin and the antipsychotic triflupromazine, where the

increase in surface area due to the combination was 10.52% for streptomycin and 12.49% for triflupromazine[28].

Table 4: Combined antifungal property fluconazole + fluoxetine

Concentration of FLUX (µg/ml)	Concentration of FLUC (µg/ml)								
	0	**0.78125**	**1.5625**	**3.125**	**6.25**	**12.5**	**25**	**50**	**100**
0	+	+	+	+	+	**+**	-	-	-
6.25	+	+	+	+	+	**+**	-	-	-
12.5	+	+	+	**+**	**+**	-	-	-	-
25	+	+	**+**	-	-	-	-	-	-
50	+	**+**	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-

+ Indicates presence of microbial growth, - indicates absence of microbial growth

Table 5: Antimircrobial activity of antibiotic and non-antibiotic drug combination against *C. albicans*

Drug combination	FIC	∑*FIC*	Interaction
Fluconazole + Propranolol	(0.125 + 0.125)	0.25	Synergism
Fluconazole + Fluoxetine	(0.125 + 0.0625)	0.1875	Synergism

These results of MIC were comparable with the experiment results obtained by Kaushiki *et al*., who screened antimicrobial potential of cardiovascular drug against eight bacterial test organisms, in which oxyfedrine HCl and obutamine were shown to have pronounced antimicrobial property. The MIC of oxyfedrine was found to be in the range of 50 – 200 µg/ml in most of the tested strains[4]. Similarly Cidalia pina-vaz *et al* study on antifungal activity of ibuprofen against *C. albicans* showed the MICs of 1-3mg/ml against 12 strains of *Candida sp*[29]. Kruszewska *et al.,* observed that tetrahydrozoline and amlodipine inhibited the growth of *S. aureus* in the concentrations of 0.05 and 3 mg/mL respectively. Famotidine in a concentration of 2 mg/mL showed the strongest activity against *E. coli*.

Hanna et al found that *Pseudomonas aeruginosa* was sensitive to cisapride, penicillamine and amidotrizoic acid with MICs of 0.05, 62 and 76 mg/mL respectively, and *C. albicans* was most susceptible to chlorpromazine and diltiazem with MICs at 20 and 26 mg/mL respectively[30]. Umaru *et al*., determined that diclofenac sodium to exhibit good antimicrobial property at a concentration of 50-100 µg/ ml[31].

These checkerboard assays indicated that MIC value of fluconazole decreased by 8 fold with propranolol and 16 fold with fluoxetine. Our results are comparable with the experimental findings of the other researchers i.e., Cidalia pina-vaz *et al* found that the MIC value of fluconazole was decreased by 2-128-fold, when fluconazole and ibuprofen were used in combination against *C. albicans*[29]. Akilandeswari *et al* observed synergism of escitalopram when combined with antibiotics like gentamycin and ciprofloxacin against *S. aureus* and there was 2 fold reduction in MIC value of gentamycin from 250 to 62.5 µg/ml and 3 fold reduction in MIC value of ciprofloxacin from 500 µg/ml to 62.5 µg/ml[21]. Pooi Yin Chung *et al*., showed that the combinations of α-amyrin and betulinic acid was found to have synergistic effect with FIC index of 0.5. The MIC for betulinic acid was reduced to 8 fold in the presence of α-amyrin. Synergy was also evident for betulinic acid in combination with methicillin and vancomycin, as the MIC of the combinations was reduced to 64 fold and 8 fold, respectively[32].

The FIC_{index} value obtained for both propranolol + fluconazole and fluoxetine + fluconazole drug combinations were found to be synergistic. When comparing antimicrobial synergy with fluconazole, the results showed that fluoxetine (∑*FIC* 0.1875) is more synergistic than propranolol (∑*FIC* 0.25). Having in mind that the rapid growth of microbial resistance mechanisms against frequently used antibiotics, a synergistic interaction might be a solution in fight with infectious diseases.

The exact mechanism by which those drug combinations showed synergistic activity is not known, though it is important to have such information, knowledge on mechanism of action is not required to determine a degree of synergy in our study, because the mass-action law based determination of synergism is mechanism- independent.

5 Conclusion

The emergence of antibiotic resistance has not yet prompted a radical revision of antibiotic utilization. Instead, to overcome

antimicrobial resistance more new antibiotics are being discovered and released to the market. This practice never ends the antimicrobial resistance development and necessitates the need of safe and innovative approach for addressing the problem effectively. In addition to non-antibiotic drugs, there are several already existing non-antibiotic approaches to the treatment and prevention of infections include probiotics, phages and phytomedicines[33].

The anti-depressant drug Fluoxetine was observed to possess in vitro antimicrobial activity against all the test microorganisms, i.e., *E. coli, S. aureus* and *C. albicans*. Thus, based on the tested spectrum of antimicrobial activity and synergy, the present study suggests that fluoxetine has a promising potential for being developed into an effective antimicrobial agent. Additional molecular and animal studies are to be performed in future to further confirm our findings and to elucidate the pharmacological basis for the antimicrobial action of fluoxetine.

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7 Conflict of interests

None

8 Authors contributions

NSB, MH and SM designed the study and carried out the literature review. MH, SM, NSB and YB collected the data. NSB and NDA prepared the manuscript and arranged in tabular form. NSB and NDA critically revised the manuscript. All authors read and approved the final manuscript.

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