Journal of Graphic Era University
Vol. 5, Issue 2, 155-163, 2017
ISSN: 0975-1416 (Print), 2456-4281 (Online)

In *Vitro* Evaluation of Anticancer Drugs as Antimicrobial Agent against the Pathogenic Enteric Bacteria

Pranjali Gupta, Pankaj Gautam*, Nishant Rai

Department of Biotechnology
Graphic Era University, Dehradun, India
*Corresponding author: gautampankaj76@gmail.com

(Received November 16, 2016; Accepted December 25, 2016)

Abstract
Drug repurposing and drug combination have been suggested as a major strategy to counter the spread of antimicrobial resistance in clinical and community settings. Drug repurposing explores the potential of existing drugs for new indications with respect to human disease. Mainstream anticancer drug paclitaxel and vinblastine were tested as potential efflux pump inhibitors in *in vitro* assays. Both the anticancer drugs failed to produce zone of inhibition on MHA plates inoculated with *Salmonella* Typhi in disk diffusion testing. MIC obtained for the two drugs were found as 80µg/ml (Paclitaxel) and 160 µg/ml (vinblastine) respectively. When tested for synergy with kanamycin and ciprofloxacin, paclitaxel and vinblastine resulted in indifference (FICI value =1 for both the antibiotics). The combination of anticancer and antibiotic drugs showed bacteriostatic nature in the absence of $3\log_{10}$ reduction in viable *Salmonella* CFUs over 24 hrs time period in time kill assay. Whereas the marginal activity was observed in EtBr cartwheel assay as compared to control. Thus *in vitro* studies paclitaxel and vinblastine cannot be repurposed as antimicrobial against *Salmonella* Typhi.

Keywords- Anticancer Drugs, Drug Repurposing, Efflux Pump Inhibitor, Multidrug Resistance, *Salmonella* Typhi.

1. Introduction

The wide spread bacterial multidrug resistance strains have necessitated the discovery of new antimicrobials (Piddock, 2006). An increased prevalence of antibiotic resistance with decreased production of new drugs has made the treatment more complicated; resulting in increased cases of morbidity and mortality round the globe (Rowe et al., 1997). Several marketed drugs have lost their efficacy over the year; they either reduce the bacterial growth when used in combination or modulate the host defence response but nowhere related to complete inhibition of bacterial growth. The combination of efflux pump inhibitors with antibiotics has been suggested for the effective management of multi drug resistance cases round the globe (Huttner et al., 2013). Repurposing of old drug may be useful tool in solving the puzzled scenario of multi drug resistance and related toxicity as the pharmacokinetics and pharmacodynamics of old existing drugs has been available to fill up the void of antimicrobial efficacy (Ahburn and Thor, 2004; Gould and Bal, 2013). In recent years anticancer drugs have under gone extensive testing as an antibacterial agent *in vitro* as well as *in vivo* (Oprea and Mestres, 2012). The Tirapazamine an anticancer drug has showed the

Journal of Graphic Era University
Vol. 5, Issue 2, 155-163, 2017
ISSN: 0975-1416 (Print), 2456-4281 (Online)

antibacterial activity (Shah et al., 2013) against bacterial strain *Escherichia coli* under anaerobic and aerobic conditions. Likewise, Celecoxib when combine with known antibiotics like kanamycin, ciprofloxacin increases the activity of bacterial strain by inhibiting its multidrug resistance efflux pumps which expels the broad range of antimicrobial out of the cell prior reaching to their intended targets (Kalle and Rizvi, 2011). Similarly norepinephrine, trimethoprim (Piddock et al., 2010), reserpine (Garvey and Piddock, 2008), has been extensively used as putative efflux pump inhibitors against Gram-negative bacteria species and plays a major role in virulence (Lomovskaya and Watkins, 2001; Li and Nikaido, 2004; Fernando and Kumar, 2013; Aygul, 2015). All the evidences from the previous year publications and the leads obtained from the *in silico* (Gupta et al., 2015) studies lead to hypothesize that the utilization of mainstream selected anticancer drug paclitaxel and vinblastine as efflux pump inhibitor molecule for *Salmonella* Typhi.

2. Material and Methods

2.1 Bacterial Strains

Bacterial strains (*Salmonella* Typhi-MTCC-733) were obtained from Institute of Microbial Technology (IMTECH) Chandigarh, India. The strain was cultured on Triple sugar ion agar (TSI) and Bismuth Sulfite Agar (BSA). Glycerol stocks of bacterial culture were stored at -20^{0}C respectively.

2.2 Anticancer and Antibiotic Drugs

Anticancer drugs; paclitaxel -T1912, vinblastine-V1377, trimethoprim-92131 were obtained from Sigma Aldrich, Germany, USA. Stock solution (5mg/ml) of paclitaxel and vinblastine were prepared in one milliliter of Dimethyl Sulfoxide (DMSO) whereas trimethoprim stock (25mg/ml) was prepared in molar ration (1:1) of ethanol: chloroform. Antibiotic; kanamycin-Cal Biochem, Germany and ciprofloxacin- HIMEDIA were obtained and prepared in sterile milli-Q water. Further, two fold dilution of each anticancer drug was used ranging from 160 µg/ml, 80 µg/ml, 400 µg/ml, 20 µg/ml and 10µg/ml whereas kanamycin and ciprofloxacin were used alone or in combination wherever required. All the solution was stored at 2-8^{0}C according to the manufacturer instruction (Sigma/HIMEDIA).

2.3 Disk Diffusion Susceptibility Test

Sterile Muller Hinton Agar (MHA-M173 HIMEDIA) plates were inoculated with *Salmonella* Typhi (1x10^5 CFU/ml) and discs containing two fold dilutions (in range of 160 µg/ml, 80 µg/ml, 40 µg/ml, 20 µg/ml and 10 µg/ml) of paclitaxel and vinblastine, were overlaid on equal space on solidified agar plates (Figure 1). Positive control (Erythromycin at 10mcg/ml) and drug vehicle DMSO discs were also included to compare the outcome after 24 hrs of incubation at 37^{0}C (Bonev et al., 2008).

Journal of Graphic Era University
Vol. 5, Issue 2, 155-163, 2017
ISSN: 0975-1416 (Print), 2456-4281 (Online)

2.4 MIC Determination by Broth Dilution Method

96-well plates were inoculated with the bacterial suspension (1 $\times 10^5$ CFU/ml) after adjusting the OD with 0.5 McFarland suspensions in Muller Hinton broth (MHB- M1657 HIMEDIA). Two fold dilution of paclitaxel and vinblastine (in range of 160 µg/ml to 10µg/ml) alone and in combination with fixed sub lethal dose of kanamycin (8µg/ml) and ciprofloxacin (1µg/ml) were given in a total volume of 200 µl in each well. Growth Control (GC) was also included and each drug; either alone or in combination, were given in triplicate. Plates were incubated for 24hrs at 37^0C. Minimum inhibitory concentration was determined as the concentration at which the visible growth was absent. Further to quantify the MIC outcome the absorbance of each well was read at OD_{600} (Sopirala et al., 2010).

FICI of drug combinations were calculated by the standard formula:
$FICI=FIC_A+FIC_B$, Where $FIC_A = MIC_A$ in combination/MIC_A alone and
Where $FIC_B = MIC_B$ in combination/MIC_B alone

Drugs interaction was interpreted as synergy if FICI ≤ 0.5; addition if FICI is between 0.5 and 1; indifference if FICI between 1 and 4 and antagonism if FICI >4.

2.5 Time Kill Assay

Salmonella Typhi (MTCC-733) (10^5 CFU/ml) was grown in multiple tubes in cation-supplemented Muller Hinton Broth (MHB) in total 10 ml volume overnight at 37ºC in orbital incubator (Sopirala et al., 2010). Each tube was treated with the combinations of anticancer and antibiotics (P+K, P+C and V+K, V+C) at two fold dilution (160µg/ml, 80 µg/ml, 40 µg/ml respectively). Aliquots were removed at different time interval starting from 0hr, 16hr and 24hrs and were plated onto the Muller Hinton Agar (MHA) plates. Before plating on MHA plates, aliquots of the bacterial culture were diluted in 0.85% NaCl solution (Olajuyigbe and Afolayan, 2012). The $\log_{10}$ CFU/ml were calculated for different time interval and graph were plotted against time and $\log_{10}$ CFU/ml (Figure 2).

2.6 EtBr Cartwheel Assay

Overexpression of bacterial Efflux Pump (EP) of *Salmonella* Typhi was induced by indole according to the described protocols (Nikaido et al., 2012) Optical density of overnight bacterial culture was adjusted (0.3OD) and induced by indole at 4mM concentration for maximum 4hrs at $\pm 35^0$C. Further, the Muller Hinton agar plates were prepared with 2.5µg/ml of concentration of Ethidium Bromide (EtBr). The test samples (induced and uninduced *Salmonella* Typhi) were swabbed as spoke on EtBr containing MHA plates. The plates were kept in dark for 16-24hrs at $\pm 35^0$C. The difference in fluorescence was observed with the help of UV gel dock (Genei ^{TM}) system and captured the images (Figure 3)

Journal of Graphic Era University
Vol. 5, Issue 2, 155-163, 2017
ISSN: 0975-1416 (Print), 2456-4281 (Online)

3. Results and Discussion

3.1 In *Vitro* Studies of Antimicrobial Properties of Anticancer Drugs Alone or In Combination Produce Non-Significant Results

The leads obtained from the computational analysis were further confirmed by conventional *in vitro* assays. In present studies we performed disk diffusion, broth micro-dilution, time kill assay and synergy testing to assess the antimicrobial potential of anticancer drugs (Reller et al., 2009; Lehar et al., 2009; Martins et al., 2013). Moreover, the inhibition studies of *Salmonella* Typhi efflux pump by anti-cancer drugs: paclitaxel and vinblastine (as prospective efflux pump inhibitors-EPIs) were carried out using EtBr cartwheel assay. Disk diffusion assay were performed according to national committee for clinical laboratory standards (Patel et al., 2015) guidelines (M02-A11, Vol 29, No-1) (NCCLS, 2015) two fold dilutions of paclitaxel and vinblastine drugs (160µg/ml to 10µg/ml each) failed to produce zone of inhibition on MHA plates inoculated with *S.* Typhi, clearly showing absence of antimicrobial effect by the drugs. Whereas control disk (erythromycin, 10mcg/ml) showed a clear visible zone of inhibition after 24 hours of incubation at 37^0C. Vehicle control (DMSO) also witnessed lack of zone of inhibition on the same plate. Zone of inhibition may depend on a number of factors including solubility, molecular weight of the test drugs; generally large molecular weight drugs register slow diffusion. Paclitaxel and vinblastine both are hydrophobic (thus may cross the lipid membrane easily) in nature and have comparable molecular weight (853.906 g/mol and 810.975 g/mol) with the positive control erythromycin (733.93 g/mol), which diffused easily and produced clear zone of inhibition in MHA plate **(**Figure 1). MIC determination for paclitaxel and vinblastine as calculated by broth micro dilution methods was found as; paclitaxel alone (80 µg/ml), paclitaxel + kanamycin (80 µg/ml), paclitaxel + ciprofloxacin (40 µg/ml), vinblastine alone (160 µg/ml), vinblastine + kanamycin (160 µg/ml), vinblastine + ciprofloxacin (160 µg/ml) as the absence of visible bacterial growth compared to growth control respectively. Various concentration of paclitaxel, when tested in combination with the sub-lethal MIC of kanamycin and ciprofloxacin, produced significant ($p<0.0001$) reduction in the visibility of bacterial suspension (Table-2). However, when used in combination with antibiotics kanamycin (sub-lethal MIC) and ciprofloxacin (sub-lethal MIC) (Table-1), paclitaxel and vinblastine produced complete inhibition of bacterial growth on EtBr containing MHA plates (Figure 3A). Surprisingly, bacterial growth appeared when antibiotics MIC were further reduced to 1/4MIC (Figure 3B). The mechanism how EtBr synergizes with antibiotic and anticancer drugs in combination is yet unknown; however, the EtBr is reported earlier to have an anti-trypanosoma activity (Chowdhury et al., 2010).

4. Conclusion

Drug repurposing and drug combinations hold great promise against drug resistant clinical pathogen *Salmonella* Typhi. In present study the main stream anti-cancer drug paclitaxel and vinblastine were explored for their potential as efflux pump inhibitor against the pathogen. Paclitaxel and vinblastine failed to register any antimicrobial effect in *in vitro* assays (disk

Journal of Graphic Era University
Vol. 5, Issue 2, 155-163, 2017
ISSN: 0975-1416 (Print), 2456-4281 (Online)

diffusion, broth micro dilution, time kill assay and synergy assay). Moreover, when tested for their potential as Efflux Pump Inhibitor (EPI), both drugs failed to produce any effect on cart wheel assay in terms of reduced fluorescence on EPI administration. Therefore, we conclude that paclitaxel and vinblastine cannot be repurposed as antimicrobial agent against human enteric bacteria.

Acknowledgment
I would like to express my deep gratitude and thankfulness to Prof. Kamal Ghanshala ji, (President, Graphic Era University, Dehradun, India) for providing financial assistance and moral support for the research work, Prof. LMS Palni (Dean, Department of Biotechnology, GEU) for encouraging scientific exploration in true spirit, Dr. Ashish Thapliyal (HOD, Biotechnology) for departmental facilities. We acknowledge the Central Molecular Research Laboratory (CMRL), SGRR institute of medical and health sciences, Dehradun for availing BSL-2 facility for *Salmonella* Typhi culture.

Figure 1. Disk diffusion susceptibility testing of paclitaxel and vinblastine

Disk showing two fold dilution of drugs concentrations (ranging from 160µg/ml to 10µg/ml). Vehicle control (DMSO) and positive control (erythromycin at- 10mcg/ml) was also included on MHA plates. Different dilutions of drugs produced no zone of inhibition, whereas positive control showing clear zone of inhibition.

Drug Alone and in Combination	MIC (µg/ml)
Paclitaxel Alone	80 µg/ml
Paclitaxel+Kanamycin	80µg/ml
Paclitaxel+Ciprofloxacin	40 µg/ml
Vinblastine Alone	160 µg/ml
Vinbalstine+Kanamycin	160µg/ml
Vinbalstine+Ciprofloxacin	160 µg/ml

Table 1. MIC of anticancer drugs (Alone and in combination)

Journal of Graphic Era University
Vol. 5, Issue 2, 155-163, 2017
ISSN: 0975-1416 (Print), 2456-4281 (Online)

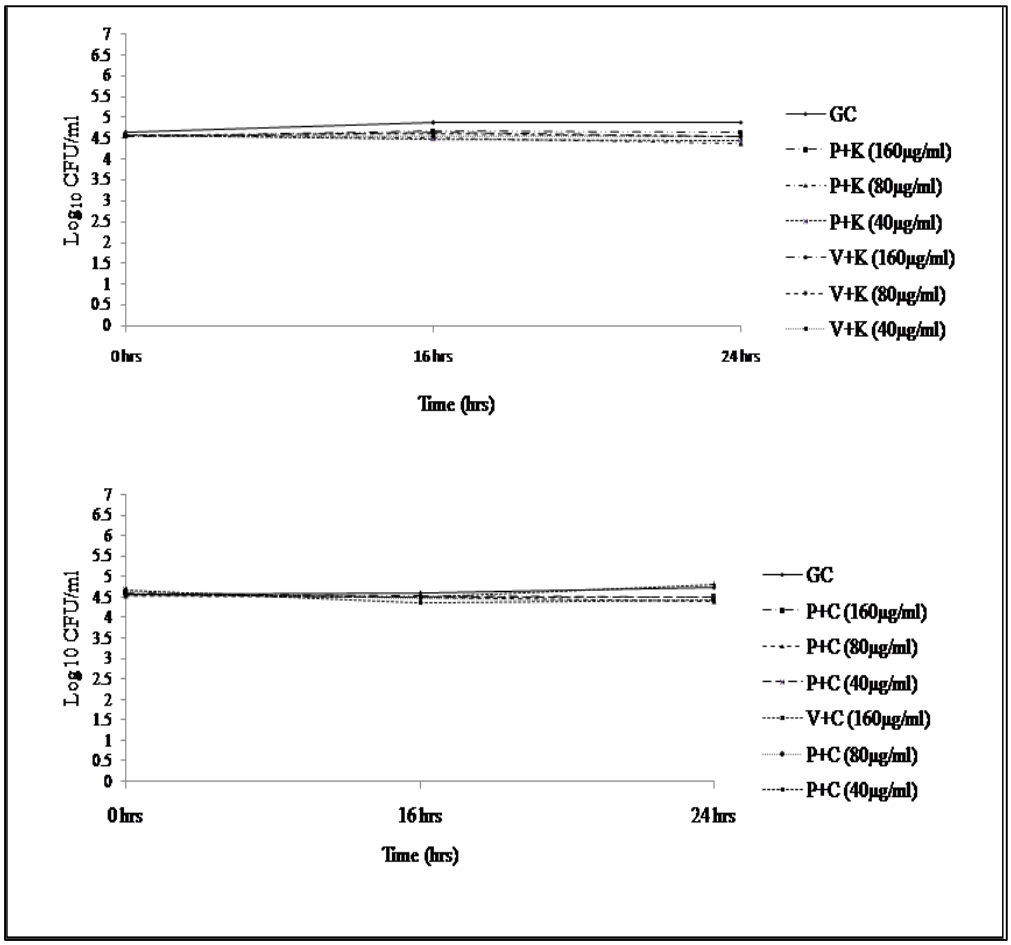

Figure 2. Log_{10}CFU values of paclitaxel and vinblastine with antibiotics (kanamycin and ciprofloxacin) at various time intervals (0 hrs, 16hrs, 24hrs) in group combination P stands for –paclitaxel, V- for vinblastine, K- for kanamycin, C-ciprofloxacin. GC- bacterial growth control, P-paclitaxel, V-vinblastine, K-kanamycin, C- ciprofloxacin

Drug combination	Concentration µg/ml	Log_{10} CFU/ml reduction as compared to GC post 16hrs	Log_{10} CFU/ml reduction as compared to GC post 24hrs	Log_{10} CFU/ml difference (24hrs-16hrs)
P+K	160 µg	0.19	0.23	0.04
	80 µg	0.37	0.53	0.16
	40µg	0.39	0.43	0.04
V+K	160 µg	0.23	0.33	0.10
	80 µg	0.27	0.34	0.07
	40µg	0.32	0.33	0.01
P+C	160 µg	0.06	0.24	0.18
	80 µg	0.09	0.33	0.24
	40µg	0.10	0.22	0.12
V+C	160 µg	0.24	0.30	0.06
	80 µg	0.13	0.30	0.17
	40µg	0.08	0.07	-0.01

Table 2. Difference of Log10CFU value between various concentrations of paclitaxel and vinblastine

Journal of Graphic Era University
Vol. 5, Issue 2, 155-163, 2017
ISSN: 0975-1416 (Print), 2456-4281 (Online)

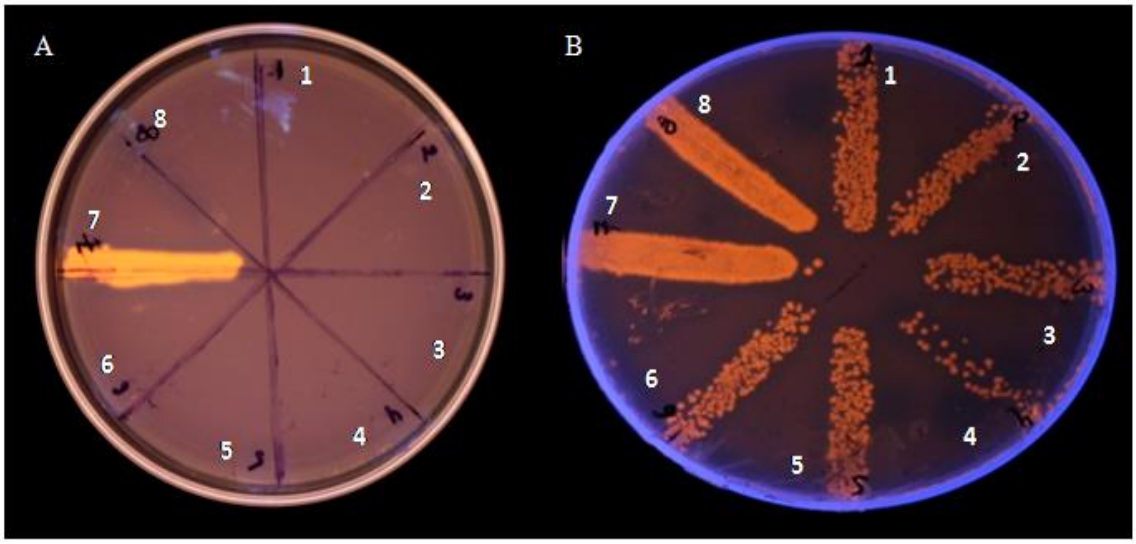

Figure 3. Plate A: Indole induced *S*.Typhi failed to grow on MHA plate when treated with paclitaxel and vinblastine (at concentration of 160µg/ml, 80µg/ml and 40µg/ml) and with sub lethal (0.5MIC) dose of kanamycin (8µg/ml) and ciprofloxacin (0.5µg/ml)
Plate B: Appearance of growth in indole induced *S*.Typhi CFUs when treated with combination of anticancer drugs (paclitaxel and vinblastine at 160 µg/ml and 80 µg/ml each) and antibiotic (at 0.25MIC). Spoke 1 consist of paclitaxel (160 µg/ml) + kanamycin (0.25MIC) Spoke 2 paclitaxel (160 µg/ml) + ciprofloxacin (0.25MIC), Spoke 3 vinblastine (160 µg/ml) + kanamycin (0.25MIC), Spoke 4 vinblastine (160 µg/ml) + ciprofloxacin (0.25MIC), Spoke 5 Paclitaxel (80 µg/ml) + ciprofloxacin (0.25MIC), Spoke 6 vinblastine (80 µg/ml) + ciprofloxacin (0.25MIC), Spoke 7 vinblastine alone at 160 µg/ml concentrartion and Spoke 8 paclitaxel alone at 160 µg/ml concentrartion

Reference

Ahburn, T. T., & Thor, K. B. (2004). Drug repositioning identifying and developing new uses for existing drug. Nature Review Drug Discovery, 3(8), 673-683.

Aygul, A. (2015). The importance of efflux systems in antibiotic resistance and efflux pump inhibitors in the management of resistance. Mikrobiyoloji Bulteni, 49 (2), 278-291.

Bonev, B., Hooper, J., & Parisot, J. (2008). Principles of assessing bacterial susceptibility to antibiotics using the agar diffusion method. Journal of Antimicrobial Chemotherapy, 61 (6), 1295-1301.

Chowdhury, R., Bakshi, R., Wang, J., Yildirir, G., Liu, B., Pappas-Brown, V., Tolun, G., Griffith, J. D., Shapiro, T. A., Jensen, R. E., & Englund, P. T. (2010). The killing of African trypanosomes by ethidium bromide. PLoS Pathogens, 6 (12), e1001226.

Patel, J. B., Cockerill, F. R., Nicolau, D. P., Bradford, P. A., Powell, M., Eliopoulos, G. M., Miller, L. A., Hindler, J. A., Swenson, J. M., Jenkins, S. G., Traczewski, M. M., Lewis, J. S., Turnidge, J. D., Limbago, B., Weinstein, M. P., & Zimmer, B. L. (2015). CLSI performance standards for antimicrobial disk susceptibility tests; approved standard twelfth edition. CLSI document M02-A12, 35(1), 1-15. Wayne, P. A.: Clinical and Laboratory Standards Institute.

Fernando, D. M., & Kumar, A. (2013). Resistance-nodulation-division multidrug efflux pumps in gram-negative bacteria: role in virulence. Antibiotics, 2(1), 163-181.

Garvey, M. I., & Piddock, L. J. V. (2008). The efflux pump inhibitor reserpine selects multidrug-resistant streptococcus pneumoniae strains that over express the ABC transporters PatA and PatB, Antimicrobial Agents and Chemotherapy, 52 (2), 1677-1685.

Gould, M., & Bal, A. M. (2013). New antibiotic agents in the pipeline and how they can help overcome microbial resistance. Virulence, 4(2), 185-191.

Gupta, P., Gautam, P. & Rai, N. (2015). Anticancer drugs as potential inhibitors of AcrAB-TolC of multidrug resistant Escherichia coli: an in silico molecular modeling and docking study. Asian Journal of Pharmaceutical and Clinical Research, 8 (1), 351-358.

Huttner, A., Harbarth, S., Carlet, J., Cosgrove, S., Goossens, H., Holmes, A., Jarlier, V., Voss, A. & Pittet, D. (2013). Antimicrobial resistance: a global view from the 2013 World Healthcare-Associated Infections Forum. Antimicrobial Resistance and Infection Control, 2(31), 2047-2994.

Kalle, A. M., & Rizvi, A. (2011). Inhibition of bacterial multidrug resistance by celecoxib, a cyclooxygenase-2 inhibitor. Antimicrobial Agents and Chemotherapy, 55(1), 439-442.

Lehar, J., Krueger, A. S., Avery, W., Heilbut, A. M., Johansen, L. M., Price, E. R., Rickles, R. J., Short III, G. F., Staunton, J. E., Jin, X., Lee, M. S., Zimmermann, G. R., & Borisy A. A. (2009). Synergistic drug combinations tend to improve therapeutically relevant selectivity. Nature Biotechnology, 27(7), 659-666.

Li, X. Z., & Nikaido, H. (2004). Efflux mediated drug resistance in bacteria. Drugs, 64 (2), 159-204.

Lomovskaya, O., & Watkins, W. (2001). Inhibition of efflux pumps as a novel approach to combat drug resistance in bacteria. Journal of Molecular and Microbiology Biotechnology, 3(2), 225-236.

Martins, M., McCusker, M. P., Viveiros, M., Couto, I., Fanning, S., Pages, J. M., & Amaral, L. (2013). A simple method for assessment of MDR bacteria for over-expressed efflux pumps. Open Microbiology, 7(1), 72-82.

Nikaido, E., Giraud, E., Baucheron, S., Yamasaki, S., Wiedemann, A., Okamoto, K., Takagi, T., Yamaguchi, A., Cloeckaert, A., & Nishino, K. (2012). Effects of indole on drug resistance and virulence of Salmonella enterica serovar Typhimurium revealed by genome-wide analyses. Gut Pathogen, 4 (1), 2-13.

Olajuyigbe, O. O., & Afolayan, A. J. (2012). In vitro antibacterial and time-kill evaluation of the Erythrina caffra Thunb. Extract against bacteria associated with diarrhoea. Scientific World Journal, 738314, 2012, 1-8.

Oprea, T. I., & Mestres, J. (2012). Drug repurposing: far beyond new targets for old drugs, American Association of Pharmaceutical Sciences, 14(4), 759-763.

Piddock, L. J. V. (2006). Multidrug resistance efflux pumps not just for resistance. Nature *Reviews* Microbiology, 4(8), 629-635.

Piddock, L. J., Garvey, M. I., Rahman, M. M., & Gibbons, S. (2010). Natural and synthetic compounds such as trimethoprim behave as inhibitors of efflux in Gram-negative bacteria. Journal of Antimicrobial Chemotherapy, 65 (6), 1215-1223.

Reller, L. B., Weinstein, M., Jorgensen, J. H., & Ferraro, M. J. (2009). Antimicrobial susceptibility testing: a review of general principles and contemporary practices. Clinical Infectious Disease, 49 (11), 1749-1755.

Rowe, B., Ward, L. R., & Threlfall, E. J. (1997). Multidrug-resistant Salmonella typhi: a worldwide epidemic. Clinical Infectious Disease, 24 (1), 106-109.

Shah, Z., Mahbuba, R., & Turcotte, B. (2013). The anticancer drug tirapazamine has antimicrobial activity against Escherichia coli, Staphylococcus aureus and Clostridium difficile. FEMS Microbiology Letters, 347(1), 61-69.

Sopirala, M. M., Mangino, J. E., Gebreyes, W. A., Biller, B., Bannerman, T., Balada-Llasat, J. M., & Pancholi, P. (2010). Synergy testing by Etest, microdilution checker board, and time-kill methods for pan-drug-resistant Acinetobacter baumannii. Antimicrobial Agents Chemotherapy, 54 (11), 4678-4683.