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Repurposing Antibiotics and B Vitamins: Exploring Cytotoxic Effects and Synergistic Interactions for Breast Cancer Treatment Cell line

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ABSTRACT

This study evaluated the potential of repurposing antibiotics and B vitamins for the treatment of breast cancer. Cytotoxicity assays were conducted on MCF-7 cells, and the promising combinations were further evaluated in a 3D model. In addition, the analysis of the gene expression of Bcl-2, P53, Caspase-3 and Bax was performed, as well as the evaluation of antioxidant properties. This comprehensive approach provides a valuable strategy for identifying potential anticancer drug candidates. Results demonstrated that norfloxacin, rifaximin, and B vitamins at a concentration of 100 ppm exhibited anticancer activity against MCF-7 breast cancer cells. This was accompanied by increased expression of p53 and Bax genes, while Bcl-2 gene expression was downregulated. These findings suggest that repurposing of antibiotics and B vitamins may offer promising therapeutic options for breast cancer. Future studies should focus on elucidating the mechanisms of action of these compounds and evaluating their efficacy and safety in preclinical and clinical trials.

Keywords: MCF-7, antibiotics, vitamin B, breast cancer, combination, 3D spheroid

1. Introduction

Breast cancer is a serious health problem that receives a lot of attention in biomedical research, it is the second leading cause of cancer-related deaths in the United States. (Anastasiadi *et al*., 2017; Ávalos-Moreno *et al*., 2020). Although the progress in cancer therapy, many treatment failures can still be attributed to the intertumoral heterogeneity, different drug resistance mechanisms, and the inherent complexity of cancer itself (Kreso and Dick, 2014). Furthermore, 90% of newly created medications are found to have low efficacy, severe side effects, or do not meet FDA regulations (Prasad *et al*., 2016). In addition to the mentioned obstacles, the astronomical cost of developing new anticancer drugs requires finding creative methods to reduce them, including discovering new uses for existing drugs (Ashburn and Thor, 2004).

Drug repurposing leads to clinical applications of clinically licensed and off-patent drugs with known targets for other indications to reduce treatment costs (Aggarwal *et al*., 2021; Correia *et al*., 2021). The repurposing of a drug requires less time to obtain the final authorization than the de novo development of a new drug after all clinical phases have already been completed. In addition, information on side effects, drug interactions and pharmacokinetics has already been completed. (Ishida *et al*., 2016).

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Several research groups published preclinical data for repurposing of non-oncology drugs for cancer including the anti-inflammatory and anti-coagulation aspirin (Tsoi *et al*., 2019), metformin with its long history in managing type 2 diabetes (Zhang *et al*., 2020), the antifungal itraconazole (Zhang *et al*., 2021), simvastatin that lessens cholesterol accumulation (Beckwitt *et al*., 2018) and niclosamide described to manage tapeworm infection(Oh *et al*., 2020). A growing list of repurposed drugs is already included in the guidelines of the National Comprehensive Cancer Network (NCCN) or the European Society for Medical Oncology (ESMO), including retinoic acid for acute promyelocytic leukemia, thalidomide for multiple myeloma, NSAIDs for desmoid tumors, and solid zoledronic acid. cancer with bone metastases. (Bouche *et al*., 2017). Furthermore, an extensive amount of effort is put towards pre-clinical and clinical research in order to find novel applications for the approved non-cancer drugs. In this context, searching PUBMED for "repurposed anti-cancer drugs" has provided 630 hits.

Chemotherapy-treated cancer patients always receive a cocktail of antimicrobial agents to compensate the chemotherapy-related immunosuppression and to protect them against the potential infections (Luyt *et al*., 2014; Tsavaris *et al*., 2002). Interestingly, it was documented that some co-treatments using chemotherapeutic agents and antimicrobial agents resulted in better results in cancer patients compared with chemotherapy alone (Pfab *et al*., 2021). Therefore, extensive research is now focused on the potential use of antimicrobial agents in oncology.

The first documented clinical trial for antibiotics repurposing is dated back to 1997, when Clarithromycin (known commercially as "BIAXIN") confirmed efficient against lung cancer (Mikasa *et al*., 1997). The cytotoxic effect of Clarithromycin was enforced by adding the proteasome inhibitor Bortezomib (Moriya *et al*., 2013). Several in vitro studies are now published to document the anticancer effect of several antibiotics including nitroxoline (Shim *et al.*, 2010), fluoroquinolones (Suresh *et al*., 2013), minocycline (Ataie-Kachoie *et al*., 2013), tigecycline (Jones *et al*., 2016), cephalosporins (Labay *et al*., 2016) and doxycycline (Scatena *et al*., 2018).

The B vitamins are water soluble small molecule compounds participating in hormone signal transduction and redox reactions essential for several metabolic pathways (Xie *et al*., 2023). Several studies documented the role of B vitamins against breast cancer. Kim *et al*., 2019 recorded role of folic acid and VitB12 in protection against BRCA-related breast cancer. Also, Pirouzpanah et al., 2019 monitored the relation between folic acid in plasma and inhibition of the proliferation, metastasis and recurrence of breast cancer. Moreover, Tabatabayi *et al*., 2020 documented the effective antitumor role of VitB6 Schiff base Mn (II) complex in breast cancer treatment. Conversely, Houghton *et al*., 2019 recorded positive correlation between the level of VitB12 in plasma and the risk of breast cancer. Finally, Arthur *et al*., 2019 found no relation between the risk of breast cancer and the level of B vitamins. Thus, the effect of B vitamins in breast cancer requires further investigation.

The effectiveness of anticancer drugs is often limited by their toxicity to normal cells and the development of drug resistance by cancer cells. Combining multiple drugs may offer a more effective approach to overcome these challenges. (Harvey, 2008).

Studies have reflected that treatment responses and gene expression profiles in spheroid multicellular 3D models are better representatives for in vivo situation, compared with 2D cultures (Riedl *et al*., 2017). In contrast to 3D models, the 2D ones lack the complex interactions between cancer cells and the extracellular matrix or tumor microenvironment which exist in the body (Rizvanov *et al*., 2010).

Therefore, the aim of the present study is to screen the potential anticancer effects of some antibiotics in combination with B vitamins against breast cancer.

2. Material and Methods

This research study was conducted in accordance with the highest ethical standards and principles, as approved by the National Research Centre's ethical committee with approval number 19/289, following Helsinki Declaration.

2.1. Antibiotics:

The antibiotics used in this study were purchased from the Egyptian pharmaceutical market. They cover a wide range of drug classes including cephalosporins, nitrofurantoin, vancomycin, quinolones, fluoroquinolones, racetams, rifamycin, tetracycline, aminoglycoside, and cotrimoxazole.

Table 1: Broad range of antibiotic drugs used in the evaluation of anticancer potential activity against breast cancer cells

Code	Drug	Family	Company
S50	Maxipime	Cefepime	SmithKline Beecham
S51	Omnicef	Cefdinir (cephalosporins)	Al-Hikma Pharmaceuticals
S52	Duraceif	Cefadroxil	SmithCline Beeclam
S53	Curisafe	Cephalosporins	Pharco Pharmaceuticals
S 55	Novactam	Vancomycin	Eipico Pharmaceutical Company
S56	Septrin	Cotrimoxazole	Aspen Pharmacare
S57	Keflex	Cephalosporins	Al-Hikma Pharmaceuticals
S58	Tarivid	Fluoroquinolones	Sanofi
S59	Amikacin	Aminoglycoside	Amoun Pharmaceutical Company
S61	Peractam	Racetams	up pharma pharmaceutical
S62	Amofluxin	Flucloxacillin	Rameda pharma
S63	Ciprofar	Ciprofloxacin	Pharco Pharmaceuticals
S64	Ciprodiazole	Quinolones and fluoroquinolones	Minapharm Pharmaceuticals
S65	Tabocine	Tetracycline	tabuk pharmaceutical company
S66	Gastrobiotic	Rifamycin	Al andalous pharmaceutical
S67	Epinor	Norfloxacin	Eipico Pharmaceutical Company

2.2. Cancer cell lines:

The human breast cancer cell line MCF-7, was kindly donated by Professor Stig Lender from the Department Oncology and Pathology at the Karolinska Institute in Stockholm, Sweden. the human breast carcinoma cell line (MCF-7).

2.3. Cytotoxicity and anticancer activity of tested drugs against breast cancer and normal cells:

DMEM-F12/10% FBS media was used to maintain the human breast cancer cell line MCF-7 and the normal human skin cell line BJ-1. The two cell lines were cultured with 5% CO2, 95% humidity, and 37°C. Subcultures of cells were treated with 0.15% trypsin.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromuro] assay: After 24 hours of seeding 10,000 MCF-7 cells per well and 50,000 BJ-1 cells per well (in 96-well plates), a final concentration of 100 ppm of the tested drugs was added in triplicate. Cells were treated with 1 μM doxorubicin as a positive control and 0.5% DMSO as a negative control for 120 hours. Cytotoxicity was determined using the MTT assay as described by Mosmann in 1983. (Mosmann, 1983). The percentage was calculated based on the following equation: [1-(av(x))/(av (NC))]*100. Where: Av: average, X: absorbance of sample, NC: absorbance of negative control. Absorbance was measured at 595nm with reference 690nm.

2.4. Calculation of IC_{50} values:

For drugs demonstrating high potency, with ≥ 70% cytotoxicity against breast cancer cells, different concentrations were prepared for dose-response studies. IC50 values were calculated using probit analysis with SPSS software. (SPSS for windows, statistical analysis software package / version 20 / 1989 SPSS Inc., Chicago, USA).

2.5. Three-dimensional cell culture model (spheroid model bioassay):

MCF-7 human breast cancer cells were seeded in DMEM-F12 complete medium (10,000-20,000 cells per well) in a 96-well plate coated with poly-HEMA to prevent adhesion to the surface. The plate was then centrifuged for 10 minutes at 3000 rpm, 4 °C. Raise it for style. 4 days, until the shape of the spheroids reaches approx. 500 microns. The language is slowly compiled and new language is added. Test samples were added to a final concentration of 100 μM each in triplicate. After 120 hours, the circles were examined under a microscope and the diameter was measured.

2.6. Gene expression analysis:

RNA extraction was followed by one-step RT-qPCR using the iScript™ One-Step RT-PCR Kit with SYBR® Green. The reaction mixture contained 2x SYBR Green®, forward and reverse primers, nuclease-free water and iScript Reverse Transcriptase in a total volume of 50 µl. Control reactions without RNA template or reverse transcriptase enzyme were included. Amplification reactions were performed using a Rotor-Gene Q Real-Time PCR System (Qiagen, Hilden, Germany) with primers targeting Bcl-2, P53, Caspase-3, Bax genes. βActin was used as housekeeping gene.

Caspase-3 F	5'- TGTTTGTGTGCTTCTGAGCC-3'
Caspase-3 R	5'- CACGCCATGTCATCATCAAC-3'
Bax F	5'-ATGTTTTCTGACGGCAACTTC3'
Bax R	5'-AGTCCAATGTCCAGCCCAT-3'
Bcl-2 F	5'-ATGTGTGTGGAGACCGTCAA– 3'
Bcl-2 R	5'GCCGTACAGTTCCACAAAGG-3'
P53 F	5'-ATGTTTTGCCAACTGGCCAAG3'
P53 R	5'TGAGCAGCGCTCATGGTG-3'
β-Actin F	5'- GTGACATCCACACCCAGAGG-3'
β-Actin R	5'- ACAGGATGTCAAAACTGCCC-3'

2.7. Protein extraction and western blot analysis:

Collected cells were frozen and then homogenized in extraction buffer containing 150mM NaCl, 50mM Tris-HCl pH 7.5, 10mM MgCl2, 1mM PMSF, 0.1% NP-40 and 1x complete protease inhibitor (Roche) using a stick "pellet pestle blue" (Sigma). antibiotics were always kept on ice and clarified by centrifugation at 13000 rpm during 10min at 4°C. Supernatants were carefully collected into a new tube and centrifuged again for 10min at 13000 rpm to remove all plant debris. This second supernatant was transferred to a new tube and the protein content was quantified by the Bradford protein assay method (Bio-Rad). Protein samples Cells were lysed and homogenized in a buffer containing NaCl, Tris-HCl, MgCl2, PMSF, NP-40, and protease inhibitors. The lysate was centrifuged to remove cell debris, and the protein concentration was measured using the Bradford assay. Protein samples were denatured, separated by SDS-PAGE, and transferred to a PVDF membrane. The membrane was probed with antibodies against p53 (Anti-p53 monoclonal antibody) (PAb 240; ab26; Abcam UK), Bcl-2(Anti-Bcl-2 monoclonal antibody (E17; ab32124; Abcam UK), Bax(Anti-Bax monoclonal antibody (E63, ab32503; Abcam UK), Caspase-3 (Anti-Caspase-3 polyclonal antibody (ab13847; Abcam UK), beta-actin (Anti-beta Actin monoclonal antibody (SP124; ab115777; Abcam UK) followed by detection with secondary antibodies. The secondary antibodies used were: Anti- Mouse IgG (Amersham Biosciences, Buckinghamshire UK) and Anti-Rabbit (GE Healthcare, Milwaukee, WI, United States).

3. Results

3.1. Cytotoxicity bioassay on MCF-7 breast cancer cell line:

The MTT test evaluated the potential anticancer effects of 16 antibiotics, either alone or in combination with B vitamins, against MCF-7 breast cancer cells at a concentration of 100 ppm. (Table 1). B vitamins showed 60% cytotoxicity, while the utilized antibiotics showed 20.1 to 86.8% cytotoxicity, recorded for S58 and S69, respectively. Combining of B vitamins with each of the utilized antibiotics produced a higher cytotoxic effect, compared with those produced by the lone application B vitamins or the corresponding antibiotic. All combinations produced a promising cytotoxic effect ranged from 85.7 to 99.4 recorded for S57 and S64, respectively. Therefore, the cytotoxic effect of 12.5, 25, 50 and 100 ppm of each combination was evaluated (Table 2). While several combinations produced total inhibition of cancer cells at 100 ppm, only the combinations containing norfloxacin and rifaximin produced a distinguished cytotoxicity at lower concentrations. Thus, the impact of norfloxacin and rifaximin either alone or in combination with B vitamins was evaluated at gene expression level.

Table 2 shows that combination of antibiotics and B vitamins significantly enhanced the anticancer activity compared to the antibiotics alone.

Table 2: % inhibition of antibiotics without B vitamin and with combinations with B vitamin at 100 ppm against MCF7

Antibiotic	% Inhibition	
	Without B vitamins	With B vitamins
S50	21.9	96.3
S51	69	91.2
S52	34.8	96.5
S53	39	91.4
S55	28.5	98.3
S56	29.3	96.9
S57	39.3	85.7
S58	20.1	90.6
S59	46.8	97.6
S61	56.1	94.9
S62	62.7	95.2
S63	67.1	91.2
S64	40	99.4
S65	77	97.8
S66	70	96.3
S67	64.1	97.07
Vit B		60

Table 3 highlights two particularly promising antibiotic and vitamin B combinations, S66+Vit B and S67+Vit B, which exhibited significantly lower IC50 values compared to the other tested combinations, indicating their superior potency against breast cancer cells.

Table 3: IC_{50} of antibiotics with combination with B vitamin

Combination	IC_{50}
S50+ Vit B	>50
S51+ Vit B	>50
S52+ Vit B	>50
S53+ Vit B	>50
S55+ Vit B	>50
S56+ Vit B	>50
S57+ Vit B	>50
S58+ Vit B	>50
S59+ Vit B	>50
S61+ Vit B	>50
S62+ Vit B	>50
S63+ Vit B	>50
S64+ Vit B	>50
S65+ Vit B	24.9
S66+ Vit B	9.2
S67+ Vit B	11.7
Vit B	>50

3.2. Assessing the impact of S66, S67, and B Vitamins on 3D cell cultures.

The images show distinct morphological changes in the 3D cell cultures treated with S66, S67, and their combinations with B vitamins compared to the control group. The spheroids treated with S66 and S67 appear smaller and more compact than the control, suggesting potential inhibitory effects

on cell growth or proliferation. The addition of B vitamins seems to have a modulating effect on the morphological changes induced by S66 and S67 as shown in Fig 1.

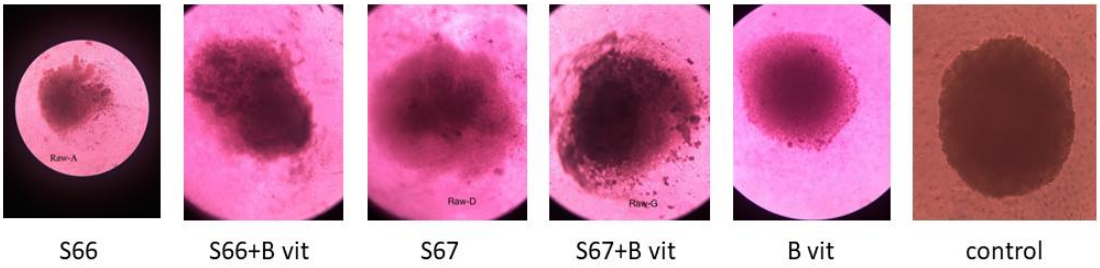

Fig. 1: Penetration capabilities of S66 and S67, as well as their combination with B vitamins within the three-dimensional cell culture model.

3.3. Gene Expression analysis

Treatment with B vitamins significantly upregulated p53 expression in MCF-7 cells, increasing levels by approximately 3-fold compared to control, both antibiotics resulted in about 30% increase in the transcript abundance of p53. On the other hand, the combined treatment of B vitamins with norfloxacin enhanced the expression to reach about 2.2 folds of control, while the combined treatment containing rifaximin up regulated the expression to reach 3.6 folds. The expression of the pro-apoptotic Bax gene was doubled compared with the untreated control following exposure to B vitamins. The lone treatment with norfloxacin and rifaximin enhanced Bax gene expression reaching 1.5 and 1.3 folds of control, respectively. The effect of B vitamins on Bax gene was not modified by combination with norfloxacin while it was enhanced following addition of rifaximin to reach about 2.8 folds of control. Concerning the anti-apoptotic Bcl-2 gene, B vitamins attenuated the expression reaching about 34% of the untreated control. Norfloxacin treatment resulted in about 20% decrease in Bcl-2 gene expression, while rifaximin resulted in 30% decrease, compared with control. The inhibitory effect of B vitamins was culminated by combination with norfloxacin, while it remained unaffected by combination with rifaximin. Caspase-3 expression reached about 3 folds of the untreated control, following treatment with B vitamins. Exposure to each of the antibiotics resulted in about 60% increase in caspase-3 expression. However, norfloxacin attenuated the promoting effect of B vitamins, while rifaximin intensified the expression to reach about 3.7 folds of the untreated control.

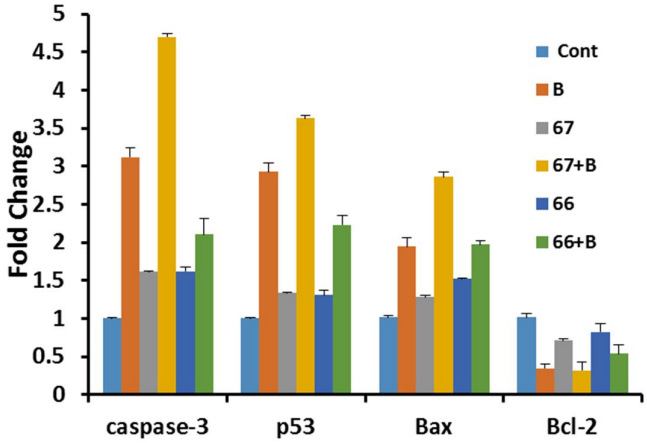

Fig. 2: Impact of antibiotics, S66 and S67, with B vitamins on apoptosis-related genes expression in MCF-7 cells.

3.4 Protein extraction and western blot analysis

The Western blot data supports the results of gene expression analysis. S66 and S67, in combination with B vitamins, may induce apoptosis in breast cancer cells through a mechanism involving the modulation of p53, Caspase-3, and Bcl-2. The combined treatment with S66 or S67 and B vitamins appears to induce apoptosis in breast cancer cells through the upregulation of p53 and pro-apoptotic proteins like Caspase-3 and Bax, while simultaneously downregulating Bcl-2.

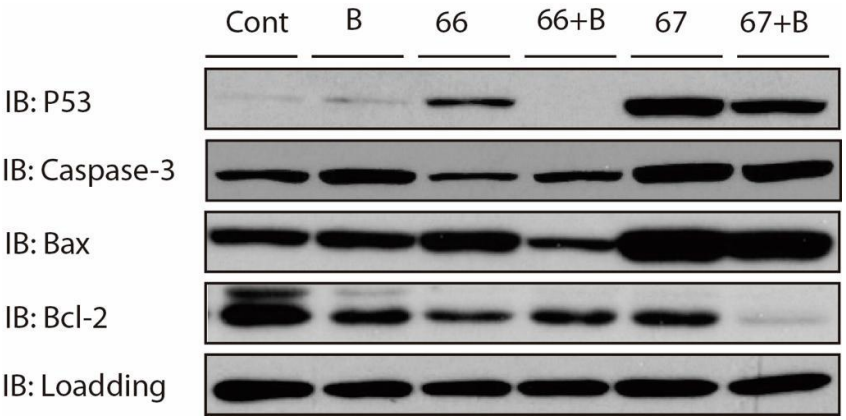

Fig 3: Western Blot analysis of anticancer effects of antibiotics, S66 and S67, with B vitamins in MCF-7.

4. Discussion

The (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction (MTT) cell proliferation assay has been widely utilized and is considered as a gold standard to evaluate drug cytotoxicity and cell viability (Kumar *et al*., 2018). Based on the MTT test, this study demonstrated the anticancer effect of 100 ppm of each of norfloxacin, rifaximin and B vitamins on the MCF-7 cancer cell line. These findings were further supported by gene expression analysis, which demonstrated increased expression of p53, Bax, and caspase-3, while Bcl-2 expression decreased. The anticancer effects of B vitamins can be attributed to their ability to stimulate the expression of the tumor suppressor p53, as previously reported in studies on various cancer cell lines. This aligns with findings by Zhang *et al*. (2014) and Cao *et al*. (2005) who observed similar effects of B6 vitamin and folic acid on p53 expression (Zhang *et al*., 2014) and the similar results reported by Cao *et al*. (2005) in the gastric mucosa in response to folic acid (vitamin B9) treatment. Activation of p53 is one of the common endogenous (mitochondrial) signals for apoptosis (Wang *et al*., 2017). It induces several genes involved in apoptosis signaling including Bax gene (Riley *et al*., 2008). As a proapoptotic member, Bax promotes apoptosis by releasing cytochrome c from the mitochondria, (Kazemi and Shahrestani, 2018). Cytochrome c participates in the formation of a protein complex known as apoptosome, the assembled complex cleaves Pro-caspase-9 to active Caspase-9. In turn, the activation of Caspase-9 activates the Procaspase- 3 into effector Caspase-3. Thus, the cascade of caspases is initiated leading to morphological manifestations of programmed cell death *(*Urbani *et al*., 2021).

The down regulation of the anti-apoptotic Bcl-2 gene recorded in the present investigation confirmed another role of B vitamins against cancer through p53 which is known to drive expression of a number of microRNA species, including miR-34 (He *et al*., 2007), that is known to target the Bcl-2 gene (Bommer *et al*., 2007).

Rifaximin is a rifamycin antimicrobial agent exerts its antibacterial effect through inhibiting the bacterial RNA synthesis through binding to the β-subunit of bacterial DNA-dependent RNA polymerase (Jiang and DuPont, 2005). It is documented to attenuate the nuclear factor (NF)-κB signaling by inhibiting IL-8 (Cheng *et al*., 2012; Flammini *et al*., 2018). The anticancer effect of rifaximin observed in the present investigation can be attributed to the mutual inhibition between NFκB and the tumor suppressor p53 (Webster and Perkins, 1999). Supporting this point of view, our results reflected increase in p53 gene expression following treatment with rifaximin. This increase was synchronized with turning on of the caspases cascade as indicated with increase in the expression

of caspase-3 gene with simultaneous up regulation of the proapoptotic Bax gene and down regulation of the anti-apoptotic Bcl-2.

Norfloxacin belonging to the synthetic antibiotics fluoroquinolones that interact with topoisomerase II-DNA complexes to inhibit helix rejoining, leading to the formation of double-stranded DNA breaks (Aldred *et al*., 2014). As demonstrated in the current results, norfloxacin exhibited cytotoxic effect underlined with alteration in the expression of p53, Bax, Bcl-2 and caspase-3 genes. Similar to rifaximin, norfloxacin-induced variations may be attributed to the potential ability of fluoroquinolones to reduce IL-8 (Reuveni *et al*., 2010).

Compared with the lone applications of B vitamins, norfloxacin and rifaximin, results of cytotoxicity reflected greater anticancer effect of combinatorial treatment composed of B vitamins and each of the utilized antibiotics. B vitamins are double edged swords; on one hand vitamins B6 and B9 enhances the tumor suppressor p53 (Cao *et al*., 2005; Zhang *et al*., 2014). On the other hand, both vitamins enhance IL-8 (Mikkelsen *et al*., 2019). Therefore, the ability of rifaximin to enhance the anticancer activity of B vitamins may be attributed to the ability of this antibiotic to reduce IL-8 (Flammini *et al*., 2018) allowing another enhancement for p53 and subsequent effects on Bax, caspase-3 and Bcl-2 genes, as recorded in our results.

The role of norfloxacin against IL-8 (Reuveni *et al*., 2010) may provide an explanation for its ability to amplify the anticancer activity of B vitamins. However, the retarding effect of norfloxacin for the impact of B vitamins on p53, caspase-3 and Bcl-2 genes remains an active area of investigation. In this context, Yadav et al. (Yadav *et al*., 2015) recorded anticancer activity for the fluoroquinolones moxifloxacin and ciprofloxacin against pancreatic cancer cells. The authors suggested modulation of apoptosis and cell survival pathway related proteins as a mechanism through which fluoroquinolones induce their apoptotic effect. They also recorded fluoroquinolones-induced apoptosis to be related to activation of extracellular signal-related kinase (ERK) independent of p53. Thus, the conflict between the ability of norfloxacin to enhance the anticancer effect of B vitamins and the ability to attenuate their impact on p53, caspase-3 and Bcl-2 genes can be explained by monitoring ERKs and survival related proteins including cellular myelocytomatosis oncogene c-(Myc) and AKT-ser 473 which is the target of our next work.

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