ANTICANCER RESEARCH *40*: 2739-2749 (2020)
doi:10.21873/anticanres.14245

Cell Cycle Arrest and Apoptotic Effect of 7-(4-(N-substituted carbamoylmethyl) piperazin-1-yl) Ciprofloxacin-derivative on HCT 116 and A549 Cancer Cells

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Abstract. *Background/Aim: Ciprofloxacin has been used as an antibiotic in the clinic for decades. Recently, ciprofloxacin and its derivatives have shown promising anti-proliferative and cytotoxic activities against several malignant cells. The aim of this study was to investigate the effect of a new derivative of ciprofloxacin on colorectal cancer (HCT116) and non-small lung carcinoma (A549) cells. Materials and Methods: Cell viability was detected by the MTT assay. Flow cytometry was used to examine the cell cycle and apoptosis. Expression of bax, bcl2, p53 and p21 was investigated by qRT-PCR and western blotting. Results: Ciprofloxacin-derivative had an anti-proliferative effect on both cell lines in a concentration-dependent manner and caused cell cycle arrest at the G_2/M phase and apoptosis. p53 and Bax proteins were overexpressed, while p21 and bcl2 gene expression was decreased after treatment with the ciprofloxacin derivative. Conclusion: This new ciprofloxacin derivative can be potentially used for the treatment of colorectal cancer and non-small lung carcinoma.*

Cancer is a significant health problem worldwide (1). Scientists around the globe extensively study various aspects of tumorigenesis to obtain novel cancer treatments, since chemotherapeutic agents show severe side effects and no selectivity. Fluoroquinolones (FQs) are a class of antibiotics that have been in clinical use for decades. They target and inhibit bacterial DNA gyrase and topoisomerase II; therefore, inhibit bacterial replication and proliferation (2). Ciprofloxacin is considered a 2nd generation class of antibiotics. Strong evidence suggests the cytotoxic effect of fluoroquinolones in general and of ciprofloxacin in particular (3). Several studies have explored alterations of ciprofloxacin and other fluoroquinolone structures to obtain new derivatives with potent cytotoxic or cytostatic activities. Structure-activity relationship studies have examined the effect of the addition of fluorine, chlorine, bromine, methyl, and oxymethyl groups (4). While the inclusion of fluorine in any position other than C-6 causes cardiotoxicity (5), the substitution of a carboxylic acid group with hydroxamic acid suppressed colon and lung cancer cell proliferation. Moreover, the introduction of hydrazine compounds to C-3 carboxylic group enhanced the cytotoxic activity against tumor cells (6, 7). It has been reported that the inclusion of a bulky functional group at C-7 position enhanced the anti-tumor activity of ciprofloxacin (8).

The 7-(4-(N-substituted carbamoylmethyl) piperazin-1 yl) derivative of ciprofloxacin belongs to the category of 4-fluoroquinolone antibiotics that are remarkably employed in the therapeutic treatment of many bacterial infections. It can inhibit the bacterial DNA gyrase enzyme, which shows antimicrobial activity. However, this drug has additionally been shown to have an effect on mammalian topoisomerase II (9). Recently, several studies have discussed in detail the role of fluoroquinolones either as single agents or in combination with anti-cancerous drugs in cell cycle arrest and cell death in numerous cancer cell lines (10-12). It has been documented that substituent insertion on the N-4-

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Key Words: Ciprofloxacin, cell cycle arrest, apoptosis, colorectal cancer, lung cancer.

piperazinyl moiety of ciprofloxacin has improved the physicochemical properties of the parent quinolone with major therapeutic changes to antitumor candidate (13-16).

p53, the tumor suppressor gene that is found mutant in almost 50% of human tumors, is a transcription factor that regulates almost 500 different genes, as a result controlling a variety of cellular processes including apoptosis, aging, cell cycle arrest, metabolism and DNA repair (17). Aberrant expression of p53 levels is critical for tumorigenesis. Thus, the goal of anticancer agents is to induce p53 expression, which increases the expression of pro-apoptotic proteins such as bax and inhibits the expression of anti-apoptotic proteins such as bcl2 to activate apoptotic cascades. p21, or the cyclin-dependent kinase (CDK) inhibitor, has been known to inhibit cyclin/CDK complexes and thus arresting the cell cycle in the G_2/M and G_1/S check points (18). Despite its role in the suppression of the cell cycle, p21 protects cells from caspase-mediated cell death. For instance, p21 can block apoptosis by binding and inhibiting the proteins involved in such process (19). According to several studies, p21 has controversial roles as it can act as an oncogene or as a tumor suppressor, which depends mainly on the drug therapy and cancer type (20). The drugs may regulate the function of p21 by affecting protein–protein interactions or gene transcription (21).

The aim of this study was to investigate the anti-proliferative and apoptotic effects of this new ciprofloxacin-derivative on the HCT 116 colorectal cancer cell line and the A549 non-small lung carcinoma cell line, and examine its ability to arrest the cell cycle and cause apoptosis *via* the p53/bax/bcl2 dependent pathway and the p21 regulated pathway.

Materials and Methods

The ciprofloxacin derivative. The tested compound was prepared by reaction of the appropriate amine with bromoacetyl bromide using potassium carbonate as a base in dichloromethane. Alkylation of ciprofloxacin with 2-bromoacetamide derivatives was achieved in acetonitrile in the presence of trimethylamine (TEA) to obtain the compound in good yield (11, 15). This derivative was identified by ^{1}H-NMR, ^{13}C-NMR and mass spectrometry as previously reported (22).

Cell culture. HCT 116 and A549 cells were obtained from American type culture collection (ATCC, Manassas, VA, USA). They were cultured in fresh Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Inc, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Biosolutions International, Melbourne, Australia), 1% penicillin-streptomycin mixture (Invitrogen, Grand Island, NY, USA), and 1% L-glutamine (Sigma-Aldrich, Inc) in a humidified 5% CO_2 atmosphere at 37˚C.

Cell viability assay. Cell viability assay was achieved using MTT [3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyltetrazolium bromide]. For both cell lines, 20,000 cells per well were seeded in triplicate in 96-well plates and allowed to grow in fresh DMEM medium for 24 h. Then, medium was replaced with fresh DMEM containing different concentrations of the target compound (0-10,000 μg/ml). After 24 h incubation, 10 μl of MTT (5 μg/ml) was added per well and incubated in the dark for 3 h at 37˚C. One hundred μl of DMSO were used to dissolve the Formazan crystals that were formed and absorbance was measured using an ELISA reader at 570 nM (23).

Annexin V assay. Apoptosis was detected using Annexin V-FITC Apoptosis Detection Kit, according to the manufacturer's instructions (Sigma-Aldrich, Eschenstr, Taufkirchen, Germany). Annexin V-FITC kit combined with flow cytometry permit quantitative determination apoptotic cells. The sites of phosphatidyl serine on cell membrane are labeled with AnnexinV-FITC. The kit includes propidium iodide (PI) to label the cellular DNA in necrotic cells. This combination permits differentiation between early apoptotic cells (annexin V positive, PI negative), late apoptotic (annexin V positive, PI positive), necrotic cells (annexin V negative, PI positive), and viable cells (annexin V negative, PI negative). The assay was performed for both untreated and treated cells with the IC_{50} of the target drug was assayed following incubation for 24 h. Cells were suspended in 1× Binding Buffer at a concentration of ~1×10^6 cells/ml. Five μl of FITC-Conjugated Annexin V and 10 μl of propidium iodide solution were added in 500 μl of cell suspensions. The cells were incubated at room temperature for exactly 10 min in the dark. Cell fluorescence was determined immediately with a flow cytometer.

Cell cycle analysis. For assessment of the cell cycle distribution, flow cytometric analysis with the use of propidium iodide was performed (24). A total of 1×10^6 cells of both control and cells treated for 24 h with the IC_{50} concentration of the target compound were collected by centrifugation, washed with phosphate buffer saline (PBS, pH=7.4, Sigma-Aldrich, Inc) and fixed with ice cold 66% ethanol. The fixed cells were washed with PBS and resuspended for 30 min in 1 mg/ml RNAse. Propidium iodide (50 μg/ml) was used to label the intracellular DNA by incubating the cells for at least 20 min at 4˚C in the dark. Then, samples were analyzed using flow cytometry. The cell cycle analysis was performed using a propidium iodide flow cytometry kit (25, 26).

RNA isolation and real-time qPCR assay. Cells were treated with the IC_{50} concentration of the target compound for 24 h. Then, total cellular RNA was extracted from the cells according to the Qiagen RNA extraction kit (Hilden, Germany) instructions. The expression of the *p53, p21, BAX*, and *Bcl2* genes was assessed by real-time-qPCR. Quantification of mRNA was achieved by utilizing the Rotor-Gene 6000 Series Software 1.7. *β-actin* was used as a control (27). Doxorubicin was used as a positive control (22). The sequences of the primers (28, 29) are mentioned in Table I.

RT-PCR was performed using the Qiagen one step RT-PCR (Qiagen). RT-PCR reactions contained 100 ng of total RNA, 1x buffer, 0.6 μM forward and reverse primers, 400 μM each of dNTP, and 2 μl enzyme mix. The conditions were as follows: 30 min at 50˚C, 15 min at 95˚C, and then 40 cycles of amplification for 1 min at 94˚C and 1 min at 55˚C followed by elongation for 1 min at 72˚C and a final extension for 10 min at 72˚C (30-32). Triplicate RT-PCR reactions were prepared for each sample. Cycle threshold (Ct) was detected for each sample, and the average Ct was calculated. In order to characterize the generated amplification mixture and to

Table I. *Primers used in real-time qPCR assay.*

Primer	Sequence of the primer
Bax	Forward: 5'-GTTTCA TCC AGG ATC GAG CAG-3'
	Reverse: 5'-CATCTT CTT CCA GAT GGT GA-3'.
Bcl-2	Forward: 5'-CCTGTG GAT GAC TGA GTA CC-3'
	Reverse: 5'-GAGACA GCC AGG AGA AAT CA-3'.
P53	Forward: 5'-CCCCTCCTGGCCCCTGTCATCTTC-3'
	Reverse: 5'-GCAGCGCCTCACAACCTCCGTCAT-3'.
P21	Forward: 5'-CATGTGGACCTGTCACTGTCTTGTA-3'
	Reverse: 5'-GAAGATCAGCCGGCGTTTG-3'
β-Actin	Forward: 5'-GTGACATCCACACCCAGAGG-3'
	Reverse: 5'-ACAGGATGTCAAAACTGCCC-3'

exclude the generation of non-specific compounds, and to avoid contamination, a melting curve analysis was performed between 60–95˚C at 1˚C intervals with the Rotor-Gene 6000 Series Software 1.7 using the SYBR Green fluorescent dye. After normalization to the β-actin expression, the target gene expression in the treated cells relative to the untreated ones was calculated.

Analysis of protein expression via western blotting. To detect the expression levels of p53, p21, bax and bcl2 proteins, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed. Cells were seeded in triplicate at a density of 2×10^5 cells per well in 6-well plates, cultured in fresh DMEM medium and then treated with or without the drug for 24 h. After washing with PBS, whole cell extracts were prepared in RIPA lysis buffer, containing 50 mM Tris–Cl, pH 7.5; 0.1% SDS, 150 mM NaCl, 0.5% sodium deoxycholate, 1 mM PMSF, and 1% Nonidet P-40, supplemented with the complete protease inhibitor cocktail (Roche, Mannheim, Germany). The protein concentration was determined according to the Bradford method (33). Cell lysates with 30 μg protein were separated by SDS-PAGE (15% acrylamide), transferred to a *Hybond*™ nylon membrane (GE Healthcare) and incubated for 1 h at room temperature in Blocking Solution. Membranes were incubated overnight at 4˚C with p53, p21, bax or bcl2 antibodies (New England Biolabs, Ipswich, MA, USA) diluted (1:1000) with PBS. Then, membranes were washed at room temperature for 30-60 min and incubated at room temperature for 1 h with the HRP-conjugated secondary antibody (New England Biolabs) diluted (1:1000) in PBS (34).

According to the manufacturer's instructions, immunoreactive proteins were detected using an enhanced chemiluminescence kit (GE Healthcare, Little Chalfont, UK) by a luminescent image analyzer (LAS-4000, Fujifilm Co., Tokyo, Japan). An antibody against β-actin (New England Biolabs, Ipswich, MA, USA) (1:1000) was used to detect β-actin, which was used as a loading control. Electrophoresis and electroblotting, using a discontinuous buffer system, were carried out in a Bio-Rad Trans-Blot SD Cell apparatus (Bio-Rad, Hercules, CA, USA). Densitometric analysis was then performed by using The Image Processing and Analysis Java (ImageJ) program. Data were normalized to β-actin levels.

Statistical analysis. Results were obtained from at least three independent experiments. Data were expressed as mean±standard deviation. Differences were analyzed by Student's *t*-test after one-way analysis of variance (ANOVA), with the use of GraphPad Prism 5 statistical software (GraphPad, La Jolla, CA, USA) and Excel software (Microsoft, Redwood, WA, USA). Differences were considered significant when the probability values (p) were less than 0.01.

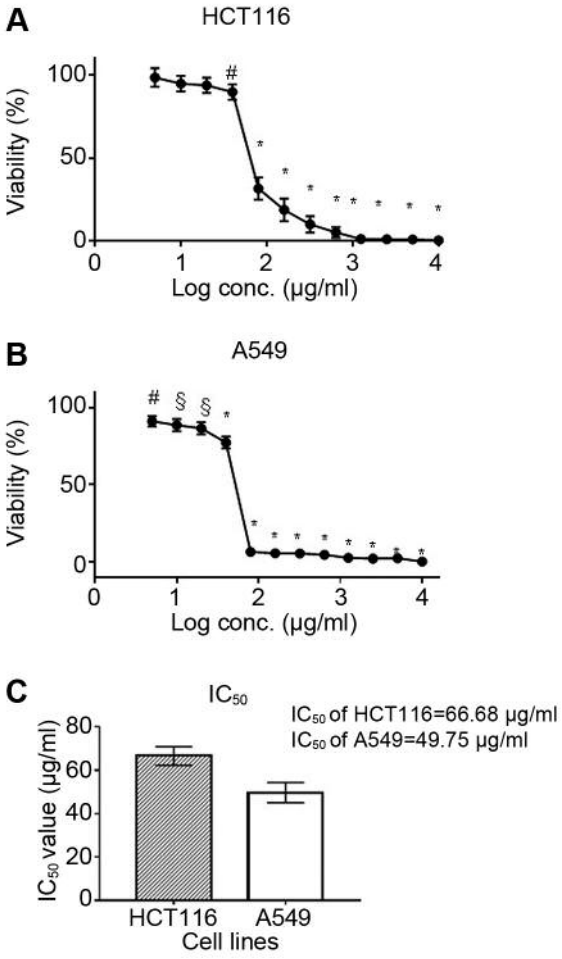

Figure 1. *Survival of HCT116 cells (A) and A549 cells (B) after 24 h treatment with different concentrations of the tested drug in fresh DMEM expressed as log concentrations. Survival of the cells was expressed as a percentage relative to that of the untreated cells, which was considered 100%. Data represent mean±SD. Significant difference was analyzed by one-way ANOVA test, where: ***$p<0.001$, §$p<0.01$, #$p<0.05$ compared to untreated cells. (C) Represents the IC_{50} values of the tested drug for HCT116 and A549 cells.*

Results

Cell viability assay. The effect of various concentrations (5-10,000 μg/ml) of the tested drug on the survival of HTC116 and A549 cells following incubation for 24 h is shown in Figure 1. The compound inhibited proliferation of HCT116 and A549 cells in a concentration-dependent manner. The target drug at the concentration of 5-30 μg/ml did not affect

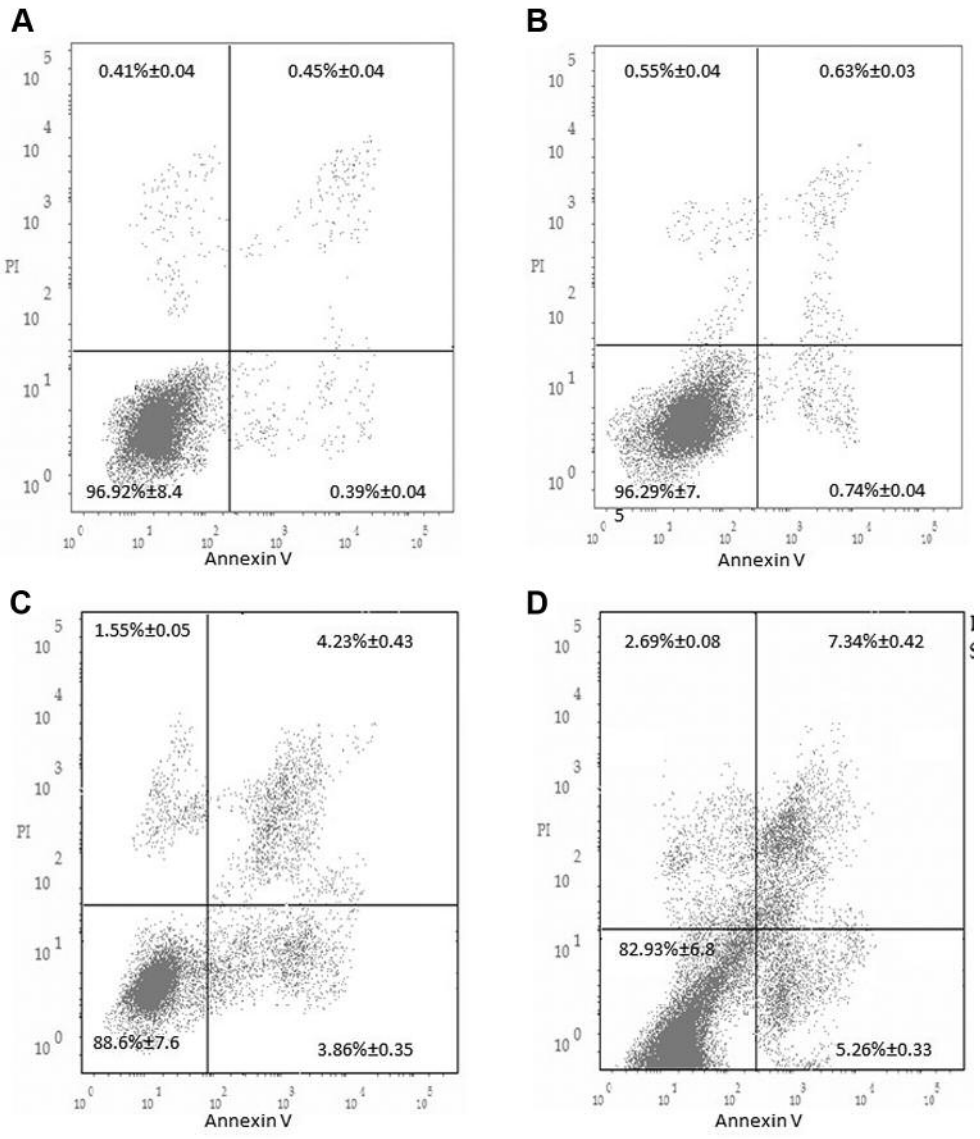

Figure 2. *Representative flowcytometric dot plots for Annexin V staining to distinguish between early apoptosis, late apoptosis and necrosis. (A) and (B) represent untreated HCT 116 and A549 cells, respectively. (C) and (D) represent HCT 116 and A549 cells, respectively, treated with the IC_{50} concentration of the target drug for 24 h for. X axis and Y axis represent FITC-conjugated Annexin V and PE-conjugated PI, respectively.*

HCT116cell proliferation significantly ($p>0.05$), however at 40-10,000 μg/ml it significantly ($p<0.001$) inhibited their proliferation, and the effect was proportional to the used concentration of the drug. Regarding A549 cells, 5 μg/ml of the drug inhibited cell proliferation significantly ($p<0.05$), in a dose-dependent manner. The IC_{50} of the drug was calculated to be 66.68±4.3 and 49.75±4.64 μg/ml for HCT116 and A549 cells, respectively, as shown in Figure 1C.

Annexin V assay using flowcytometry. Cell apoptosis was estimated using an Annexin V assay. Exposure of HCT116 and A549 cells to the IC_{50} concentration of the examined compound for 24 h, induced apoptosis. The proportion of early apoptotic (Annexin V-positive/PI-negative) cells significantly ($p<0.001$) increased from 0.39±0.04% (untreated cells) to 3.86±0.35% (treated cells) and from 0.74±0.04% (untreated cells) to 5.26±0.33% (treated) for HCT116 and A549, respectively (Figure 2). It also significantly increased the percentage of late apoptotic (Annexin V-positive/PI-positive) cells from 0.45±0.04% (untreated cells) to 4.23±0.43% (treated cells) and from 0.63±0.03% (untreated cells) to 7.34±0.42% (treated cells) in HCT116 and A549 cells, respectively ($p<0.001$).

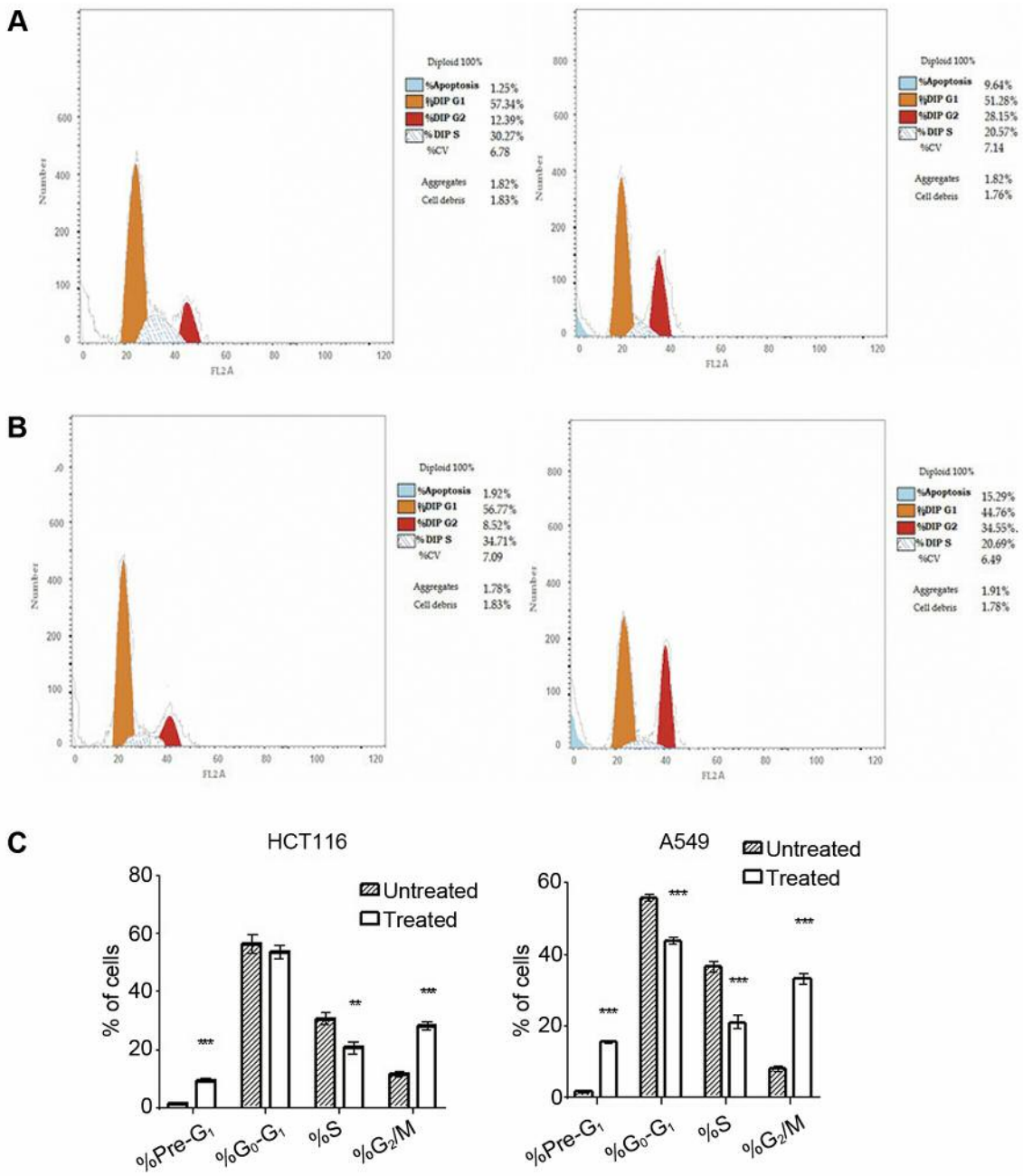

Figure 3. *Cell cycle phase distribution of untreated cells and cells treated with the IC_{50} concentration of the drug for 24 h as determined by flow cytometric analysis. (A) Representative dot plots for cell cycle distribution after PI staining for untreated (left) and treated (right) HCT116 cells. (B) Representative dot plots for cell cycle distribution after PI staining for untreated (left) and treated (right) A549 cells. (C) The percentage of untreated and treated cells in the various cell cycle phases. *p<0.05, **p<0.01, ***p<0.001.*

Cell cycle analysis using flowcytometry. As changes in cell cycle kinetics often precede the induction of apoptosis, we examined the cell cycle changes in the presence of the target drug in both cell lines. In conformity to our annexin V results, we observed a significant (p<0.001) increase in the $preG_1$ peak and cell cycle arrest at the G_2/M phase after treatment with the drug in both cell lines (Figure 3).

Expression of Bax, bcl2, p53, and p21 genes. In this study, *bax, bcl2, p53* and *p21* genes were amplified and normalized to *β-actin* by using quantitative real time PCR, as shown in Figure 4, while doxorubicin was used as a positive control. When compared to the untreated cells, expression of the *bax* gene was significantly (p<0.001) increased 3.04±0.26 and 7.01±0.37-fold in HCT116 and A549 cells treated with the

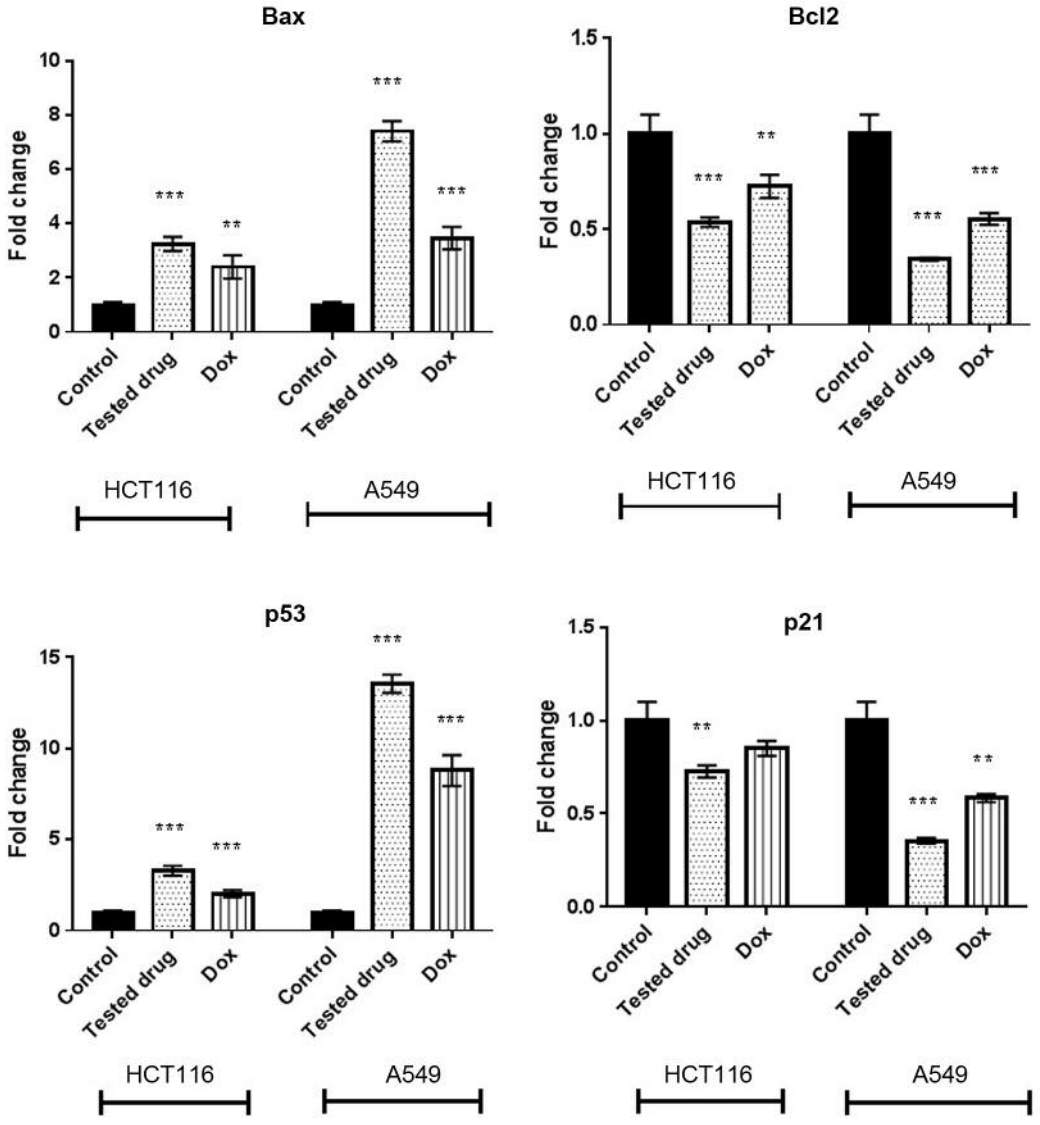

Figure 4. *Expression of bax, bcl2, p53 and p21 genes was determined by quantitative real time PCR. Relative gene expression in HCT116 and A549 treated cells with the IC_{50} concentration of the drug for 24 h compared to untreated cells. Doxorubicin was used as positive control. Expression was normalized to the corresponding β-actin gene expression. Bars represent mean±SD. Significant difference was analyzed by one-way ANOVA test, where: ***p<0.001, **p<0.01 compared to untreated cells.*

drug, respectively. *bcl2* expression was significantly (p<0.001) decreased in HCT116 and A549 treated cells 0.511±0.02 and 0.35±0.008-fold, respectively, when compared to untreated cells.

P53 gene expression was significantly (p<0.001) increased in HCT116 and A549 treated cells 3.201±0.266 and 13.89±0.49-fold, respectively, when compared to untreated cells.

Regarding the *p21* gene, its expression in HCT116 treated cells was significantly (p<0.01) decreased 0.7±0.03-fold, when compared to untreated cells. In A549 treated cells, compared to untreated cells, its expression was significantly (p<0.001) decreased 0.36±0.014-fold.

Western blotting. The protein expression of bax, bcl2, p53 and p21 in HCT116 and A549 cells treated without or with the IC_{50} concentration of the drug for 24 h is shown in Figure 5A. After normalization to the control β-actin protein expression, the comparison between treated and untreated cells, showed different expression levels in both cell lines (Figure 5B).

Bax protein expression was significantly increased (p<0.001) in HCT116 and A549 treated cells 1.285±0.028

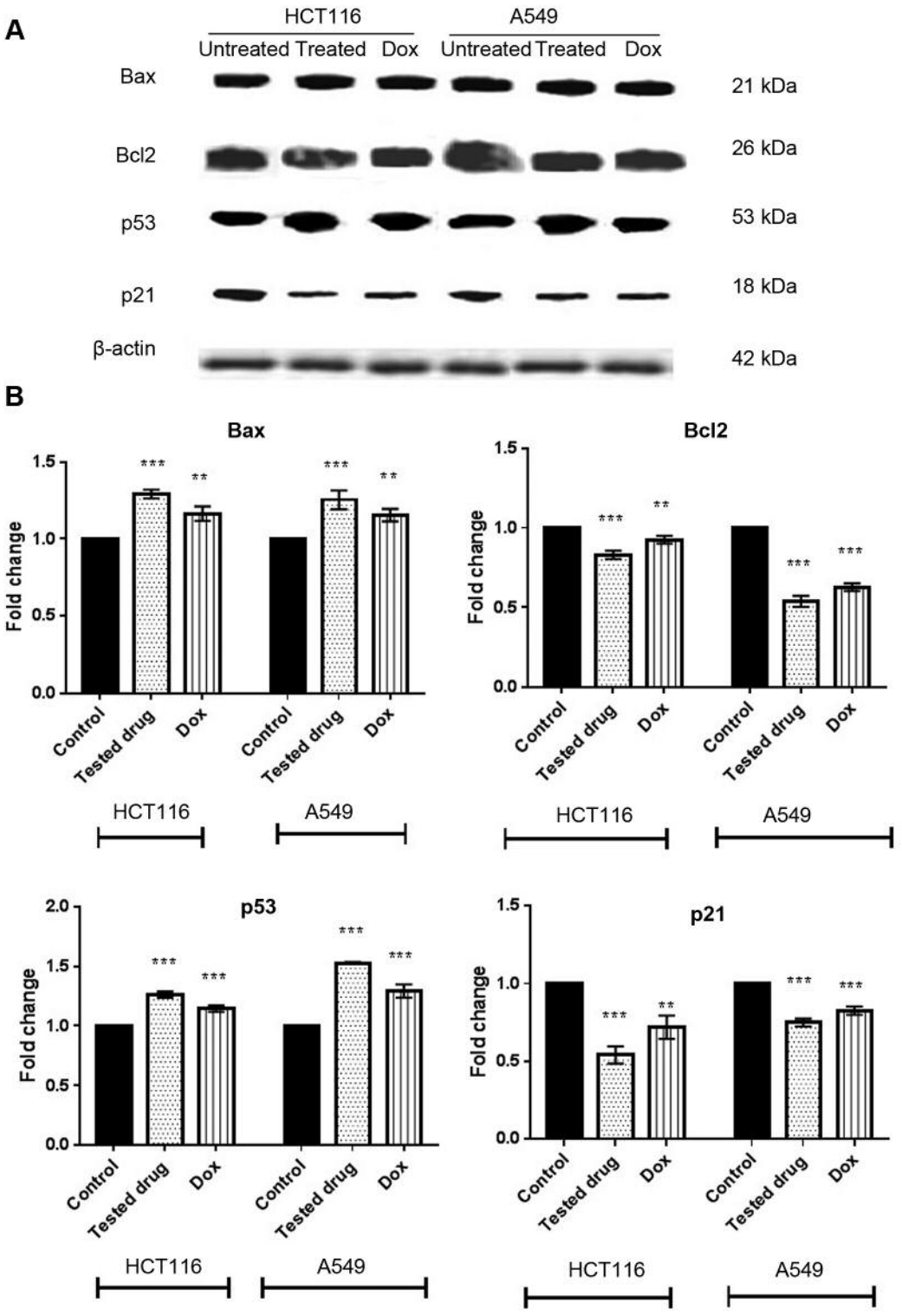

Figure 5. *Expression of bax, bcl2, p53 and p21 proteins in untreated cells and cells treated with the IC_{50} concentration of the drug for 24 h was determined by western blot. (A) Representative western blots for bax, bcl2, p53 and p21 proteins in both cells. β-actin was used as internal loading control. Doxorubicin was used as positive control. (B) Relative protein expression in HCT116 and A549 treated cells with the IC50 concentration of the drug for 24 h in comparison to untreated cells. Expression was normalized to the corresponding β-actin protein expression. Doxorubicin was used as positive control at 1 μM. Bars represent mean±SD. Significant difference was analyzed by one-way ANOVA test, where: ***p<0.001, **p<0.01 compared to untreated cells.*

and 1.225±0.06-fold, respectively, when compared to the untreated cells. Regarding bcl2 protein expression, it was significantly decreased ($p<0.001$) in both treated cells 0.837±0.025 and 0.535±0.035-fold, respectively, when compared to untreated cells.

For p53 protein expression, results showed a significant increase ($p<0.001$) in HCT116 and A549 treated cells 1.235±0.026 and 1.535±0.008-fold, respectively, when compared to the untreated cells. In HCT 116 treated cells, p21 protein expression was significantly decreased ($p<0.001$) 0.536±0.054-fold compared to the untreated cells, while its expression in A549 treated cells was significantly decreased ($p<0.01$) 0.771±0.025-fold compared to the untreated cell.

Discussion

Fluoroquinolones are synthetic broad-spectrum antibiotics that are widely used against various infections. Certain members of these antibiotics exhibit antitumor activity *in vitro* in various cancer cell lines and also *in vivo* (11, 35). This anti-tumor activity has been linked to the inhibition of the eukaryotic analogue of DNA gyrase and topoisomerase IIα activity (36). The anti-tumor activity of topoisomerase inhibitors may potentially occur *via* the inhibition of mitochondrial DNA synthesis, which subsequently induces mitochondrial injury, inhibition of the respiratory chain and depletion of ATP. Energy depletion favors apoptosis, as it may cause cell cycle arrest in the G_2/M and/or S-phases (37-40).

In this study, we investigated the effect of 7-(4-(N-substituted carbamoylmethyl) piperazin-1-yl) ciprofloxacin derivative in colorectal cancer (HCT116) and non-small lung cancer (A549) cell lines. Several studies have indicated the cytotoxic effect of the parent drug ciprofloxacin on different cancer cells. Nevertheless, the alteration and modification of the ciprofloxacin structure aimed to increase its cytotoxicity and anti-proliferative activity; this is in agreement with Mohammed and his colleagues who have shown that substitution of the phenyl ring of phenylacetanimide residues at para position is critical for its cytotoxic activity. However, the replacement of the phenyl ring with the cyclohexyl moiety adequately preserved the cytotoxicity. Also, their compound caused a concentration dependent cell cycle arrest, elevated the levels of p53 and p21 proteins and decreased the levels of cyclin B1 and Cdc2 proteins (22).

Our results showed a promising growth inhibitory effect of the tested drug in both cell lines. The A549 cells were more sensitive to the drug, as a significant inhibition of proliferation and cell viability occurred with as little as 5 μg/ml, while HCT116 cells showed a significant inhibition from 40 μg/ml upwards. Also, the IC_{50} of the drug was lower in A549 than in HCT116 cells. Furthermore, we analyzed the cell cycle of both cell lines before and after treatment with the tested drug and found a significant increase in the preG_1 peak and cell growth arrest at the G_2/M phase, similarly to the findings of Herold and his colleagues (41). It has been demonstrated that cell cycle regulation is a method of regulating cell growth (37, 42). Therefore, anticancer therapies can be used to block the cancer cell cycle. Yadav and Varshney reported that the growth inhibitory effect of fluoroquinolones on the survival of pancreatic cancer cells by inducing cell death and S-phase arrest was mediated by a decrease in p21, p27, cyclin-A, CDK2 and cyclin-E expression (11). It was indicated that, in pancreatic cancer cells, induction of apoptosis and S-phase arrest mediated the anti-proliferative effect of moxifloxacin and ciprofloxacin. Ciprofloxacin was more effective in inducing apoptosis than moxifloxacin (38). Thus, cancer cell death through apoptosis and cell cycle arrest is a therapeutic goal in cancer therapy; it removes cancer cells from the body in a strictly organized manner, which is achieved by our tested drug.

In addition, we analyzed the levels of different genes and protein to understand the mechanism by which this compound exerts its antitumor activity. Treatment of HCT 116 and A549 cell lines with the drug resulted in increased expression of *p53* and *Bax* genes which are pro-apoptotic genes and decreased expression of *p21* and *bcl2* genes which are anti-apoptotic genes. These results were confirmed by western blot analysis; there was increased expression of p53 and Bax proteins in both cell lines and decreased expression of p21 and bcl2 protein in cells treated with the new derivative when compared to untreated cells. While pro-apoptotic proteins promote apoptosis by releasing cytochrome *c* from mitochondria, anti-apoptotic proteins prevent apoptosis by delaying such release. The apoptosis is regulated by the balance between the pro- and anti-apoptotic proteins and not the total amount, this regulates the onset of apoptosis (40, 43). The tumor suppressor gene, p53 participates into certain cellular processes that involve apoptosis. p53 protein binds to DNA at specific sequences and regulates transcription of different pro-apoptotic genes such as Bax (44). Cytochrome *c* is released from mitochondria in response to signals by the pro-apoptotic proteins which inactivate the anti-apoptotic genes such as those of the Bcl-2 family. The p53 protein may induce either growth arrest at G1 or G2 or direct cell death by reacting to injured DNA. It also protects the cells from double strand DNA damage by a relatively similar mechanism (45). Beberok and his colleagues investigated the effect of ciprofloxacin on p53, bax and bcl2 signaling pathway in breast cancer. Their results demonstrated that ciprofloxacin significantly, in a concentration-dependent manner, enhanced Bax expression. By contrast, the same concentrations of ciprofloxacin suppressed the expression of bcl-2. Consequently, the Bax/Bcl-2 ratio was significantly increased, in breast cancer cells, following treatment with ciprofloxacin (36).

It has been shown that one of the key responses of drug-induced DNA damage is the expression of p53 that, *via* the intrinsic mitochondrial pathway, leads to apoptosis induction (46). Our results demonstrated that the new derivative induces apoptosis in HCT116 and A549 cells, which was accompanied by p53 expression up-regulation; this suggests that p53 pathway activation may be involved. It was also observed that it mediates the up-regulation of Bax and down-regulation of Bcl-2 expression, thus inducing apoptosis. Therefore, it may stimulate the opening of the mitochondrial permeability transition pores *via* the Bax/Bcl-2-dependent pathway (36).

In this study, p21 expression levels were investigated in HCT116 and A549 cell lines following drug treatment. We showed that our ciprofloxacin derivative can significantly down regulated p21 mRNA and protein. We have shown that the significant down-regulation of p21 could be also the result of degradation of p21 by the 26S proteasome (47) and that its degradation coincided with the induction of apoptotic cell death (48). This p21 down-regulation was accompanied by a significantly increased G_2 phase arrest, decreased proliferation and increased apoptosis, likely to allow removal of cells with damaged DNA. These results are in agreement with those of others who indicated p21 protein degradation after drug treatment, in various cell types (49). Although down regulation of p21 protein expression in some cells induces apoptosis, its loss, in other cells, can promote carcinogenesis and increase cellular proliferation (50). Evidently, p21 suppresses the p53-dependent apoptosis (50). There are two pathways that are activated following DNA damage; p53-mediated cell death or p21-dependent cell cycle arrest. It is not totally clear how a cell selects between the two, but sometimes high expression of p21 prevents p53-dependent cell suicide and cell cycle arrest. It is clear that p21 inhibits apoptosis, which may or may not be p53-dependent. Reactive oxygen species and DNA damage activate p53-dependent apoptosis or p53-dependent activation of p21 which shields the cells from cell death. Our hypothesis is that decreased p21 expression by the new derivative improves the apoptotic effect of p53.

The present study is the first to indicate that this newly synthesized 7-(4-(N-substituted carbamoylmethyl) piperazin-1 yl) ciprofloxacin-derivative inhibits human HCT116 and A549 cell viability, induces G_2/M phase cell cycle arrest and induces apoptosis through up-regulation of p53 and Bax expression and down-regulation of Bcl-2 and p21 expression. The results of the present study may provide a novel perspective into the therapeutic properties of this new ciprofloxacin-derivative to treat colorectal cancer (HCT116) and non-small lung carcinoma (A549). Further research is required to improve understanding of the molecular mechanism that underlies the anti-proliferative effect of this derivative.

Conflicts of Interest

The Authors declare no potential conflicts of interest regarding this study.

Authors' Contributions

M.F. designed and supervised the research, evaluated the data and wrote the manuscript. R.A. was involved in data curation, formal analysis and writing the original draft of the manuscript. M.H.N. assisted with experimental design and writing the original draft of the manuscript. G.A. and M.A. conceived the study and were involved in project administration. All Authors read and approved the final article.

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Received March 12, 2020
Revised March 24, 2020
Accepted March 27, 2020