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Curcumin inhibits the replication of rotavirus *in vitro*

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Summary. – Rotavirus is the most important etiological agent of infectious diarrhea in children under 5 years of age with more than 125,000 deaths occurring annually worldwide. The present study aims to determine the effect of curcumin, a natural polyphenol compound, on rotavirus in a cell culture model. The anti-viral activity of curcumin was evaluated by reverse-transcriptase quantitative PCR (RT-qPCR), $TCID_{50}$, and western blot techniques to assess CC_{50} in curcumin-treated MA104 cells as well as EC_{50} and SI within the infected MA104 cell line. Our findings supported that curcumin exerted an inhibitory influence against rotavirus in a dose-dependent manner and decreased the viral titer and VP6 expression by ~99% at a concentration of 30 μM ($p < 0.001$). To the best of our knowledge, this is the first study that reveals curcumin ability to inhibit rotavirus infection *in vitro*. The mechanism of such anti-viral activity may be due to its direct effect on the virus-cell attachment or downregulation of the structural VP6 protein in the early stages of infection; however, much research is still needed to validate this claim.

Keywords: curcumin; rotavirus; RT-qPCR; in vitro; anti-rotavirus agent

Introduction

Rotavirus is an icosahedral, non-enveloped virus belonging to the *Reoviridae* family. It has a triple-layered capsid structure with eleven segments of double-stranded RNA molecules in its core. This pathogen is responsible for more than 125,000 annual deaths worldwide and counts for the most important etiological agent of infectious diarrhea in children under five years of age. It is critical to note that rotavirus may cause severe and sometimes fatal complications such as acute gastroenteritis with or without vomiting, fever, nausea, abdominal pain, and cramps. The high percentage of rotavirus-related deaths in most developing countries (>80%) has made this pathogen a public health problem; hence a strong need for more effective prophylactic and therapeutic measures is already being demanded in those affected countries (Troeger *et al*., 2018a,b).

Rotarix and RotaTeq have been known as two potent rotavirus vaccines against particular strains since they can strongly induce the production of neutralizing antibodies; however, the reason for their lack of sufficient effectiveness in some developing countries has persisted as a challenge and requires more investigation (Parashar, 2016). According to a study, the immunogenicity of rotavirus vaccines also varies between infants in developed and developing countries with a trend of reduced immunogenicity in countries with the lowest level of

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Abbreviations: CC_{50} = 50% cytotoxicity concentration; EC_{50} = 50% effective concentration; HRV4 = human rotavirus strain RV4; MOI = multiplicity of infection; RT-qPCR = reverse transcriptase quantitative PCR; SI = selectivity index; $TCID_{50}$ = 50% tissue culture infectious dose

income. These variations in efficacy are not surprising given the differences in the epidemiology of rotavirus disease between the developing and developed countries and could reflect differences in force of infection, host genetic backgrounds and characteristics (e.g. level of nourishment, breastfeeding, etc.), the circulating strain, genotypes patterns, and the presence of other enteric pathogens (Jiang *et al*., 2010).

Regarding the treatment field, various anti-rotavirus drugs such as chemicals, probiotics, immunoglobulins, and natural products have been developed worldwide, even though their efficacy, side effects, and cost of production remain to be addressed (Grandy *et al*., 2010; Téllez *et al*., 2015; Superti *et al*., 1997). Overall, there is currently no clinically approved anti-rotavirus drug, and the gold standard treatment for patients with acute gastroenteritis mostly depends on retrieving of the lost fluids and electrolytes (Crawford *et al*., 2017). With that in mind, alternative approaches for developing new anti-viral agents must be taken into consideration as a prime priority.

Curcumin (diferuloylmethane), a natural polyphenolic compound also known as turmeric, is one of the best examples of plant derivatives with enormous pharmaceutical applications like antioxidant, anti-cancer, anti-diabetic, anti-microbial, and anti-viral activities (Hatcher *et al*., 2008). It is an active substance from the traditional herb *Curcuma longa*, which is commonly used as a spice and coloring ingredient in food. Curcumin has been shown to be active against different human viral pathogens, such as influenza A virus (IAV), Zika virus (ZIKV), Chikungunya virus (CHIKV), Japanese encephalitis virus (JEV), enterovirus 71 (EV71), hepatitis C virus (HBC), and recently the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) among the others (Anggakusuma *et al*., 2014; Dutta *et al*., 2009; Chen *et al*., 2010; Mounce *et al*., 2017; Ardebili *et al*., 2021). These studies revealed that curcumin limited viral infections by targeting its replication mechanism directly or through modulation of the host cell's signaling pathways including NF-κB, PI3K-AKT, and inflammatory mediators as well as cellular transcription/translation machinery (Moghadamtousi *et al*., 2014; Mathew and Hsu, 2018). Therefore, curcumin is likely to have an inhibitory impact on the function of viral proteins and prevent rotavirus infections.

In the present study, we investigated how effectively curcumin inhibits the replication of rotavirus in MA104 cells with minimum complications by evaluating the 50% cytotoxicity concentration (CC_{50}), 50% effective concentration (EC_{50}), and subsequently the selectivity index (SI). CC_{50} was defined as the concentration of curcumin in which 50% of curcumin-treated cells lost their viability when compared to untreated cells, and EC_{50} was specified as the concentration of curcumin in which the propagation of rotaviruses within infected cells is reduced by 50% when compared to the virus control. We used 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide cell proliferation assay (MTT) to determine CC_{50}, and a selection of different assays including RT-qPCR, median tissue culture infectious dose ($TCID_{50}$), and western blot to assess EC_{50}. Also, SI was calculated as CC_{50} divided by EC_{50} to provide us with an insight into the toxicity/therapeutic ratio of curcumin.

Materials and Methods

Cells and virus strain. MA104 cell line (derived from African green monkey kidney) was purchased (cell bank of Pasteur Institute, Tehran, Iran) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM pyruvate, high glucose, 100 U/ml penicillin, and 100 µg/ml streptomycin and incubated at 37°C in 5% CO_2 for 24 h. Human rotavirus strain RV4 (HRV4) was purchased from the European Collection of Authenticated Cell Cultures (ECACC) and activated by 5 µg/ml trypsin at 37°C for 1 h. The confluent MA104 cell monolayer was prewashed with phosphate-buffered saline (PBS) and inoculated with the activated HRV4 at a multiplicity of infection (MOI) of 1 PFU/cell. The inoculum was removed 1h later and replaced with a fresh FBS-free medium. After 5 days, when cytopathic effect (CPE) progressed to >80%, the lysate (product of cell lysis) was frozen and thawed twice, and the supernatant was harvested as virus stock. Viral titer was subsequently determined by $TCID_{50}$, and virus stock was stored at -70°C.

Compound preparation. Curcumin (Sigma-Aldrich, Missouri, USA) was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 100 mM and stored at -80°C. Working concentrations were diluted with DMEM prior to the downstream applications.

Cytotoxicity test. MTT assay was used to assess the cell viability as described previously (Ferrari *et al*., 1990). MA104 cells were seeded onto 96-well plates at a concentration of 1×10^4 cells/well and incubated at 37°C in 5% CO_2 for 24 h. At 50–60% confluency, cells were treated with an increasing concentration of curcumin dissolved in DMSO (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µM), and 48 h later, 100 µl of MTT (Sigma-Aldrich, Missouri, USA) at a concentration of 5 µg/ml was added to each well. The plates were incubated at 37°C in 5% CO_2 for 4 h, and cytotoxicity was colorimetrically determined by comparing the survival rate of curcumin-treated cells (<100% absorbance) versus those treated by DMSO (100% absorbance) as a negative control. Finally, to measure the CC_{50} value, concentration-effect curves were plotted and interpreted using nonlinear regression analysis, and means ± standard deviation (SD) of three independent experiments.

Inhibitory analysis. MA104 cells were seeded onto 48-well plates at a concentration of 4×10^4/well 24h before the initiation of anti-viral experiments which were performed in three different ways; 1) pre-treatment, 2) co-treatment, and 3) post-treatment. From this point on, curcumin concentrations used in all three methods were chosen to be those remarkably below that of CC_{50} value to avoid cell cytotoxicity, and EC_{50} was obtained by comparing the anti-viral effect of those non-toxic concentrations of curcumin with DMSO as a negative control. Besides, to augment the accuracy of our experiments, we performed two independent repetitions of the assays mentioned below for each selected concentration. Also, a set of wells was used as a mock control filled with only DMEM and the cultured cells to assess the possible antiviral feature of DMSO. In the pre-treatment assay, serially diluted concentrations of curcumin were added to the cell-contained wells and incubated at room temperature (RT) for 2 h prior to HRV4 inoculation (MOI = 2 PFU/cell). In the co-treatment method, a premix of HRV4 (MOI = 2 PFU/cell) and the mentioned dilutions of curcumin were added to the cells and incubated at RT for the entire length of the experiment. For the post-treatment approach, cells were first infected with HRV4 (MOI = 2 PFU/cell), and 2 h later, fresh DMEM complemented with the same non-toxic dilutions of curcumin was replaced with the infected culture medium. Finally, the anti-viral activity of each curcumin concentration was evaluated by RT-qPCR, $TCID_{50}$, and western blot in all three treatment methods.

RNA isolation and RT-qPCR. TRIzol (Invitrogen, Massachusetts, USA) was used to extract total RNA from the three groups of infected MA104 cells treated by curcumin in "Inhibitory analysis". To quantify the genome copies of HRV4 in each individual approach, one-step RT-qPCR was carried out using a forward (5-CGAGTCTTCGACATGGAG-3) and a reverse (5-CAACGTGAATGAAGCTG-3) primer as well as the QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) for detection of VP6 gene. Thermal cycle (ABI, Step-One Plus) protocol was as follows: 1 cycle at 50°C for 30 min, 1 cycle at 95°C for 2 min, and 40 cycles at 95°C for 10 sec and 60°C for 30 sec. Quantification cycles (C_q) were plotted for each treatment method, and the absolute number of RNA copies was determined by comparing the obtained C_q values against an extrapolated standard curve generated from a 10-fold serial dilution of a known viral RNA concentration (Yin *et al*., 2015). In the end, the number of viral RNA copies in the infected cells treated by a particular concentration of curcumin that was half the viral RNA copies in DMSO-treated cells was considered as EC_{50} in this assay, and SI was subsequently calculated.

$TCID_{50}$ assay. Results concerning the copy number of viral RNAs assessed by RT-qPCR may mislead to the quantification of both infectious and non-infectious viruses. So, we incorporated $TCID_{50}$ assay as well to enrich our results by taking into account the titer of progenitor virions only. To achieve this purpose, at a concentration of 2×10^4/well, MA104 cells were seeded onto 96-well plates containing DMEM and incubated overnight at 37°C with 5% CO_2. Ascending dilutions of viral suspension (10^{-4} to 10^{-9}) from the 48-well plates that we used in "Inhibitory analysis" were prepared in FBS-free DMEM and added to the cells in the 96-well plates. The inoculum was replaced with a fresh culture medium supplemented with 2% FBS 1h later, and all the plates were incubated at 37°C in a CO_2 incubator for 5 days. Afterward, CPE was observed under the light microscope (Olympus BX41TF, Japan), and virus titration was calculated and expressed as $TCID_{50}$/ml using the Reed-Muench formula (Atkinson, 1961). Finally, the concentration of curcumin with a $TCID_{50}$ value equal to half of $TCID_{50}$ in DMSO-treated cells was considered as our EC_{50} in this assay, and SI was calculated.

Western blot. Curcumin-treated cells subjected to "Inhibitory analysis" were scraped and collected at an appropriate time point, then washed twice with PBS, and disrupted in RIPA lysis buffer (10 mM Tris-HCl, pH 7.8, 100 mM NaCl, 0.1% NP-40, and 10% anti-protease cocktail) (Roche, Indiana, USA) for 30 min on ice. The lysates were centrifuged at 800 x *g*, and concentrations of proteins were normalized by Bradford assay (Bradford, 1976). Samples were then resuspended in Laemmli sample buffer with 50 mM dithiothreitol, boiled for 5 min, and proteins were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transference onto a nitrocellulose membrane (He, 2011). The membrane was blocked with 5% non-fat skimmed milk containing Tris-buffered saline (TBS) plus 0.05% Tween 20 (TBS-T) for 2 h at RT. The membrane was then incubated at RT for 2–3 h with a primary antibody (rabbit anti-VP6 serum, Sigma-Aldrich, Missouri, USA) diluted in TBS-T (1:2,000). Also, a rabbit monoclonal antibody against beta-actin was taken as an internal control throughout the experiments. Subsequently, the membrane was washed by TBS-T at least four times and incubated for 1h at RT with a diluted goat anti-rabbit antibody (Sigma-Aldrich, Missouri, USA) (1:3,000) conjugated with horseradish peroxidase as our secondary antibody. Finally, for semi-quantitative analysis of proteins, signals were developed using the enhanced chemiluminescence (ECL) kit (Amersham, Buckinghamshire, UK) according to the manufacturer's recommendation.

Statistical analysis. All data were recorded and calculated by Prism software version 6, and results were tabulated based on the differences between means, which had been statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The statistical significance was defined as * = $p<0.05$, ** = $p<0.01$, and *** = $p<0.001$.

Results

Evaluation of curcumin cytotoxicity

In the present study, non-toxic concentrations of curcumin were defined as those that allow >90% cell viability. Results showed that MA104 cells responded to curcumin

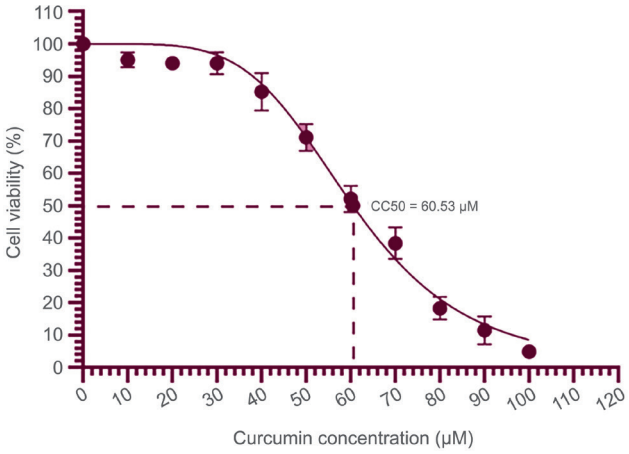

Fig. 1

Curcumin cytotoxicity assay
Cell viability was assessed by MTT assay and expressed as a percentage relative to DMSO-treated cells. Values represent the mean ± SD of three independent experiments, and CC_{50} was determined to lay in the concentration of 60.53 μM by nonlinear regression analysis.

in a dose-dependent manner, and the maximum concentration that still could be considered as non-toxic was 30 μM leading to 94.2% cell viability compared to DMSO control. In concentrations of 40, 50, 60, 70, 80, 90, and 100 μM, the viability was reduced to 83.03%, 70.62%, 52.66%, 37.58%, 17.92%, 10.69%, and 4.20% respectively, thus CC_{50} was obtained at 60.53 μM, and concentrations up to 30 μM were examined to determine EC_{50} and its inhibitory effect against HRV4 (Fig. 1).

Reduction of HRV4 titer and genome copies in curcumin-treated cells

As described earlier, the three non-toxic concentrations of curcumin (10, 20, and 30 μM) were independently examined twice (a total of 6 experiments for each assay), and the means were co-analyzed in relation to DMSO control. According to our findings, $TCID_{50}$ results denoted a progressive decline in the virus titer as concentrations of curcumin gradually increased, and EC_{50} for this assay

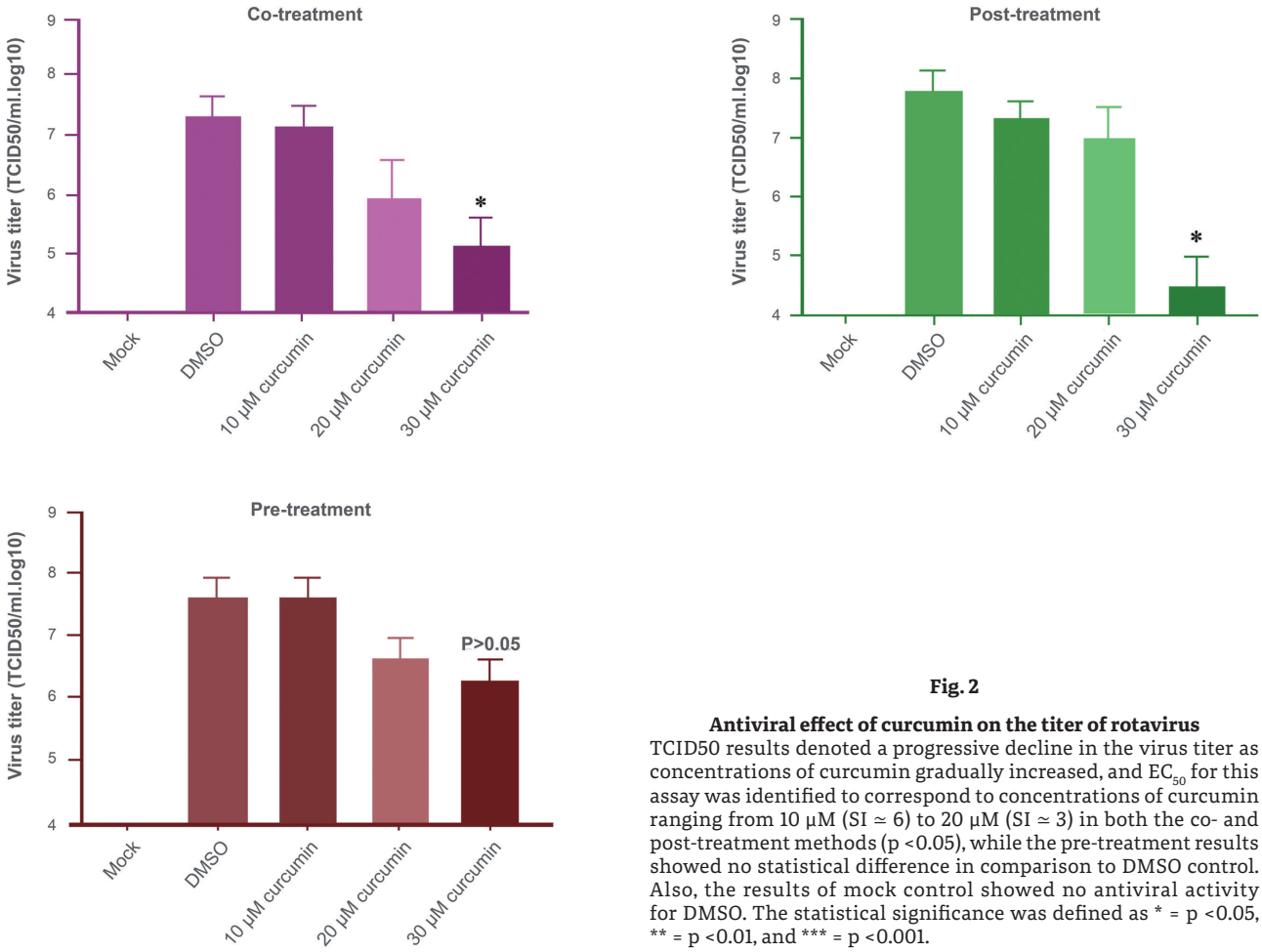

Fig. 2

Antiviral effect of curcumin on the titer of rotavirus
TCID50 results denoted a progressive decline in the virus titer as concentrations of curcumin gradually increased, and EC_{50} for this assay was identified to correspond to concentrations of curcumin ranging from 10 μM (SI ≃ 6) to 20 μM (SI ≃ 3) in both the co- and post-treatment methods ($p < 0.05$), while the pre-treatment results showed no statistical difference in comparison to DMSO control. Also, the results of mock control showed no antiviral activity for DMSO. The statistical significance was defined as * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

Table 1. Summary of EC_{50} (μM) data for curcumin in RT-qPCR and $TCID_{50}$

Treatment method	EC_{50} and SI in different assays					
	RT-qPCR		$TCID_{50}$		Western blot	
	EC_{50}	SI	EC_{50}	SI	EC_{50}	SI
Pre-treatment	17.98 ± 3.83	~3.36	45.77 ± 8.61	~1.32	-	-
Co-treatment	10.97 ± 1.86	~5.51	29.78 ± 5.90	~2.03	-	-
Post-treatment	12.40 ± 1.41	~4.88	25.85 ± 10.88	~2.34	-	-

was identified to correspond to concentrations of curcumin ranging from 10 μM (SI 6) to 20 μM (SI ≃ 3) in both the co- and post-treatment methods ($p < 0.05$), while the pre-treatment results showed no statistical difference in comparison to DMSO control (Fig. 2).

Results from RT-qPCR also revealed that the pre-treatment ($p < 0.004$), co-treatment ($p < 0.0001$), and post-treatment ($p < 0.0006$) of MA104 cells with the three non-toxic concentrations of curcumin diminished the viral RNA copies when compared to DMSO control (Fig. 3). We found out that curcumin significantly repressed the replication of HRV4 *in vitro* in a dose-dependent manner, and 30 μM concentration reduced the copies of the viral genome by 0.7 log ($p < 0.0001$), while 10 μM concentration exerted such an inhibitory influence by only 0.2 log ($p < 0.015$) (Fig. 3). In accordance with the RT-qPCR results, EC_{50} was observed to be <10.97 ± 1.86 μM (SI ≃ 6) and <12.40 ± 1.41 μM (SI ≃ 5) in the co- and post-treatment

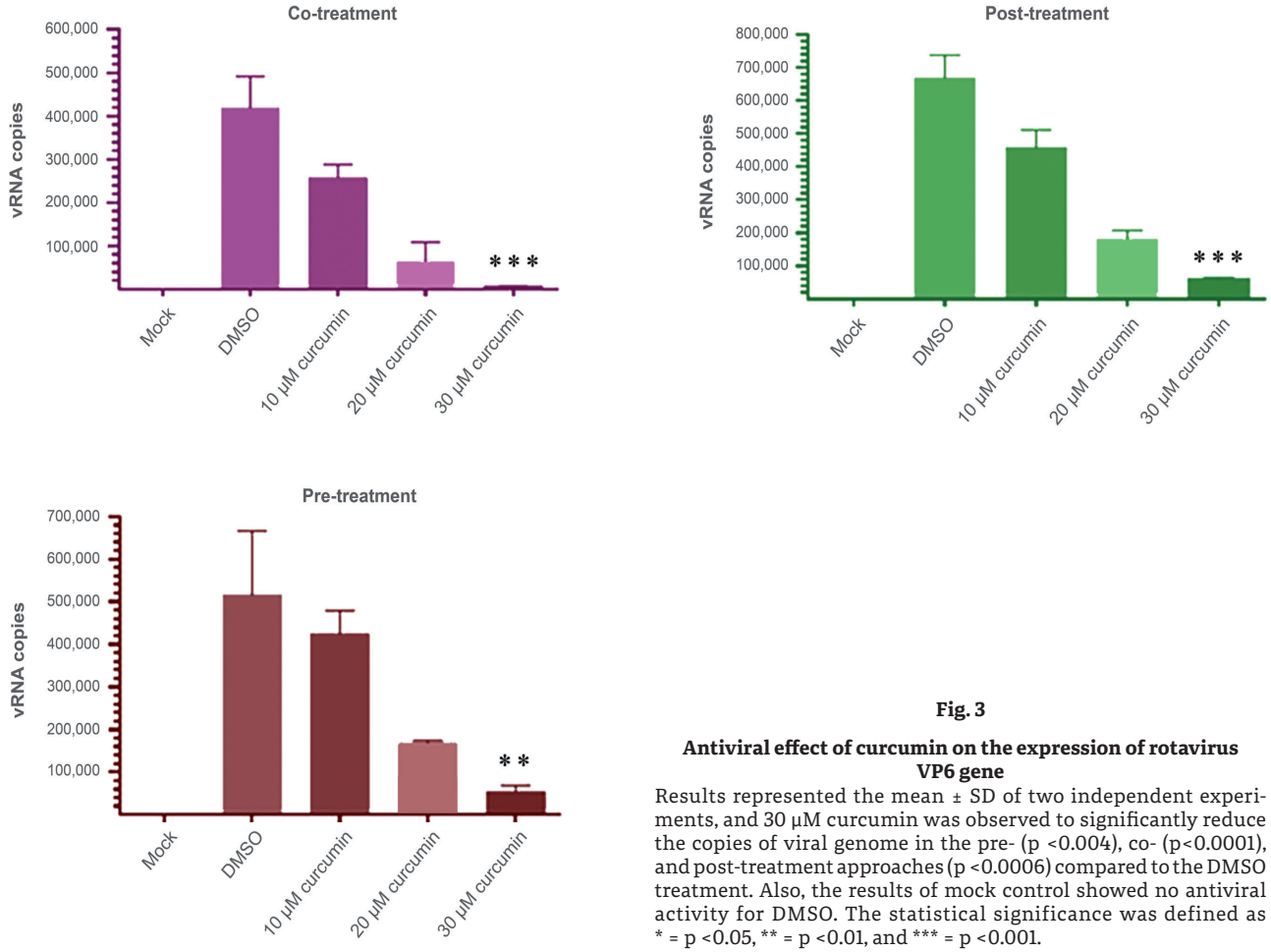

Fig. 3

Antiviral effect of curcumin on the expression of rotavirus VP6 gene

Results represented the mean ± SD of two independent experiments, and 30 μM curcumin was observed to significantly reduce the copies of viral genome in the pre- ($p < 0.004$), co- ($p < 0.0001$), and post-treatment approaches ($p < 0.0006$) compared to the DMSO treatment. Also, the results of mock control showed no antiviral activity for DMSO. The statistical significance was defined as * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

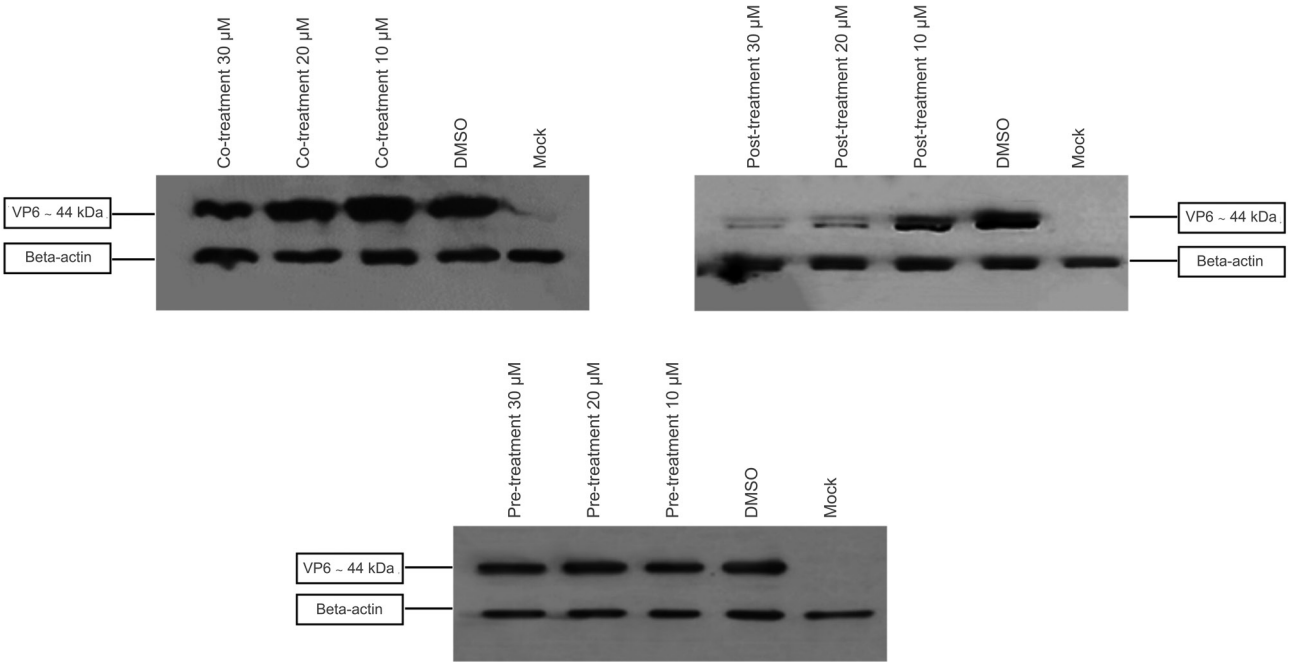

Fig. 4

Antiviral effect of curcumin on the production of VP6 protein

Unlike the pre-treatment approach, the intensity of the VP6 specific band in the co- and post-treatment conditions dramatically declined as the concentration of curcumin was elevated from 10 µM to 30 µM. Also, the results of mock control showed no antiviral activity for DMSO. Blots were developed using an ECL kit and X-ray film.

approaches respectively, whereas the pre-treatment resulted in a less statistically valid difference compared to DMSO control (Table 1). Besides, our findings showed no antiviral characteristic for DMSO when compared to the mock control (Fig. 1).

Effect of curcumin on the production levels of VP6 protein

Samples from each treatment method were collected and processed for western blot analysis to visualize VP6 protein. It was observed that the formation and thickness of protein bands were negatively co-related to the concentration of curcumin, and the intensity of the VP6 specific band dramatically reduced when the concentration of curcumin rose from 10 µM to 30 µM in the co- and post-treatment methods. In contrast, no significant difference was noticed between the pre-treatment results and that of the DMSO control (Fig. 4). Speaking of which, it should be noted that the difference between the intensity of bands was discerned by X-ray films, and no CCD digital imaging was used to provide an EC_{50} and SI value.

Discussion

In recent years, numerous studies have been conducted to investigate the inhibitory effects of various natural compounds against rotavirus in cell culture models. It should be noted that the prophylactic or therapeutic application of such compounds has been among the engaging fields of research to inspect their anti-viral properties against different strains of rotavirus for clinical purposes (Sun *et al.*, 2016; Alfajaro *et al.*, 2012; Kim *et al.*, 2012). Based on the evidence we collected from our study, it appears that curcumin has enough of the pharmaceutical potential to be used against rotavirus infections if the appropriate dose, route, and method of administration are taken into consideration.

Our results suggest that curcumin suppresses rotavirus infection in MA104 cells by probably interfering with viral mechanisms needed to facilitate the protein expression and replication of HRV4 as the total number of genome copies and the transcription of VP6 protein both declined when curcumin concentration was moderately elevated from 10 µM to 30 µM. We assume that curcumin may not have significant prophylactic features against HRV4 since our results mostly supported

the anti-rotaviral activity of this component only in the co- and post-treatment approaches. It is worth noting that concentrations of curcumin beyond the CC_{50} limit will end up in cytotoxicity, and great care must be taken concerning the determination of the most effective dose with a minimum level of toxic complications.

Curcumin has also been shown to have anti-viral features against other human viral pathogens, including influenza virus, rift valley fever virus (RVFV), HBV, and HCV (Anggakusuma *et al*., 2014; Dutta *et al*., 2009; Chen *et al*., 2010). In a study, it was demonstrated that curcumin decreased influenza viral load with an EC_{50} of approximately 0.47 μM and an SI of 92.5 using plaque reduction assays within infected MDCK cells. Accordingly, a time-of-addition assay revealed a direct inhibitory effect of this natural compound on H1N1 and H6N1 infectivity by blocking the hemagglutinin (HA) protein. The authors indicated that curcumin disturbed the virus-cell attachment which led to the suppression of influenza virus infection; however, the interaction between curcumin and HA protein or other viral surface proteins remained to be discovered (Chen *et al*., 2010). In another study, the respiratory syncytial virus (RSV) has been shown to be sensitive to this agent, and concentrations of curcumin ranging from 5 μM to 15 μM could reduce the expression of N protein by 50% to 90% respectively. This study asserted that curcumin prevented RSV replication and its budding from human nasal epithelial cells by regulating the cellular factors, including NF-κB, eIF-2α dephosphorylation, proteasome, COX2, and epithelial barrier molecules. Although these findings were in favor of our results, they still do not provide any evidence on the direct anti-viral effect of curcumin on RSV itself in the infected lung cells (Obata *et al*., 2013). In a recent study, it was reported that curcumin had significant inhibitory activity against the replication of enterovirus 71 as a non-enveloped virus. They showed that when cells were exposed to 40 μM curcumin shortly after infection with enterovirus 71, viral proteins and titer dropped to a value about 106-fold smaller in comparison to non-treated infected cells (Huang *et al*., 2018). Similarly, our *in vitro* experiments revealed for the first time that curcumin had some anti-rotaviral properties that interfere with at least the viral transcription and replication mechanisms in a multi-step viral growth system at a low MOI (0.1 $TCID_{50}$/cell). We presume that the other strains of rotavirus might be sensitive to curcumin as well; nevertheless, further research is still needed to investigate the case and clarify the specific viral site(s) that curcumin would target.

The limitations of the present study (opposed to some others) were the lack of a one-step viral growth system at a high MOI (2-10 $TCID_{50}$/cell) (Kim and Chang, 2011; Shen *et al*., 2013) and the absence of a thorough investigation of the structure of viroplasm by confocal microscopy or western blot analysis. Regarding the last case though, it has been reported that the viroplasm contains non-structural proteins such as NSP2 and NSP5 along with the structural proteins, including VP1, VP2, VP3 as well as VP6 (La Frazia *et al*., 2013) which was the particular one we analyzed in the present study.

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