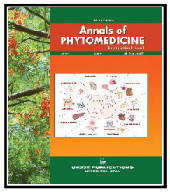
Annals of Phytomedicine 13(2): 484-490, 2024

DOI: http://dx.doi.org/10.54085/ap.2024.13.2.48

Annals of Phytomedicine: An International Journal
http://www.ukaazpublications.com/publications/index.php

Print ISSN : 2278-9839 Online ISSN : 2393-9885



Original Article : Open Access

In vitro antiviral and cytotoxicity assessment of curcumin, eugenol and azadirachtin in foot and mouth diseases virus in BHK-21 cells

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Article Info

Article history
Received 17 October 2024
Revised 4 December 2024
Accepted 5 December 2024
Published Online 30 December 2024

Keywords
Antiviral
Curcumin
Eugenol
Azadirachtin
Cytotoxicity
FMD

Abstract

"Foot and mouth disease" (FMD) is a transmissible ailment impacting animals with cloven foot. Curcumin, eugenol, and azadirachtin, the principal phytochemicals of *Curcuma longa*, *Ocimum sanctum*, and *Azadirachta indica*, respectively, have demonstrated antiviral efficacy against numerous viruses. This study evaluated the phytochemicals' cytotoxic effects in BHK-21 cell by using trypan blue (dye exclusion method). Evaluation of phytochemicals' antiviral potentiality against the FMD virus ('O'-serotype) was assessed by MTT assay. Phytochemical were prepared in serial two-fold dilutions (1.95 to 1000 µg/ml) using DMEM media and $10^{6.25}$ $TCID_{50}$ of the FMD virus were utilized. The average CC_{50} value of curcumin, eugenol and azadirachtin were found to be 285.79 ± 3.02 µg/ml, 204.53 ± 1.22 µg/ml and 129.37 ± 1.04 µg/ml, respectively. Curcumin and eugenol showed better virus inhibitory activity with IC_{50} values of 25.53 ± 1.48 µg/ml and 25.57 ± 1.25 µg/ml, respectively, than azadirachtin (39.37 ± 0.10 µg/ml). Azadirachtin showed narrower selectivity with a selective index (SI) value of 3.27 ± 0.04 than curcumin (11.22 ± 0.60) and eugenol (8.07 ± 0.71), respectively.

1. Introduction

In India, "Foot and mouth disease" (FMD) is a transmissible ailment impacting animals with cloven foot (Singh *et al*., 2013). Serotypes of FMDV are 'O', 'A', and 'Asia-1', among which serotype 'O' is the most commonly encountered in FMD outbreaks across India. There is growing interest among the scientific community in plant-based remedies against various diseases and a good number of researches have been undertaken for the use of plants to treat humans and animal illnesses (Kumar *et al.*, 2017). Globally, individuals are increasingly embracing traditional medicine for their health requirements (Sundarrajan, 2023). In India, the earliest references (3500-1800 BC) of use of some medicinal plants were mentioned in Rigveda (Nagaiah, 2022). Numerous studies have indicated that various traditional medicinal plants exhibit significant antiviral properties against viruses (Yasmin *et al*., 2020). A range of biological phytochemicals are found in plants, such as terpenes, terpenoids, and aromatic molecules, *etc*. (Bakkali *et al*., 2008). However, the antiviral effects of only a small number of these compounds have been studied (Jassim and Naji, 2003). The antiviral effects of these phytochemicals against different pathogens position these compounds as potential candidates for establishment of novel antiviral remedies generated from natural resources targeting sensitive viruses (Zorofchian Moghadamtousi *et al*., 2013). In the current context, it is vital to research on plants containing bioactive chemicals for the generation of novel antiviral medications (Senthilkumar *et al*., 2021). Curcumin, eugenol and azadirachtin have also showed antiviral efficacy against several viruses (Rechtman *et al*., 2010; Shojania and *in vitro* O'Neil, 2010). The purpose of this investigation was to assess the antiviral efficacy and cytotoxic effects of azadirachtin, eugenol, and curcumin in the BHK-21 cell line against the FMD virus.

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2. Materials and Methods

2.1 Cell culture and cytotoxicity assay in BHK-21 cells (CC_{50})

The method of cell culture conditions and cytotoxicity (CC_{50}) of plant phytochemicals was carried out in accordance with Baruah *et al*. (2021).

2.2 FMD virus

FMD virus (FMDV), Serotype 'O' was received from ICAR-AICRP on FMD, Microbiology Department, CVSc, AAU, Khanapara, Assam. The 70-80% confluent BHK-21 monolayer was inoculated with 0.1 ml of FMD virus in 25 cm^2 cell culture flask. Then the flask was incubated at 37°C for 1 h in 5% CO_2 for adsorption of virus, and it was gently shaken after every 15-20 min to ensure uniform adsorption. After observing cytopathic effect (CPE) of the virus in BHK-21 cells, maintenance media (10 ml) containing 5% FBS was added and the flask was incubated at 37°C. After development of 70-80% CPE, cells were harvested by repeated freezing and thawing of the flask. The harvested cells in the flasks were transferred to cryovials and stored at -20°C until they were needed again.

2.3 Preparation of phytochemical solutions

Curcumin, eugenol, and azadirachtin were diluted to a concentration of 10 mM in dimethyl sulfoxide (DMSO) and further dilution was made with phosphate-buffer saline solution (PBS) to 100 mM. To

prepare the cells for infection, the diluted solutions of curcumin, eugenol, and azadirachtin were added directly to the media 2 h prior to infection. To measure viral titers, the FMDV inoculum was introduced to cells maintained in serum-free DMEM supplemented with phytochemicals. The mixture was incubated then at 37°C for 8 h before the MTT test. The virus was also cultured in DMEM without serum and without phytochemicals, which served as a control group. The percentage of the virus survivability was estimated by comparing the titer of the incubated virus with the phytochemicals to the titers of the virus without phytochemicals (Mounce-Bryan *et al*., 2017).

2.4 Estimation of $TCID_{50}$

The viral infectivity assay ($TCID_{50}$) of the inoculated FMD virus was determined following standardized procedures of the Virology laboratory manual (Burleson *et al*., 1992). The experiment was conducted using 24-well cell culture plates (Nunc) with a 10-fold dilution series of the virus. BHK-21 cells were then seeded into a plate containing 96-well cells and incubated (37°C) in 5% CO_2 for 24-48 h, until they reached 70-80% confluency. Five sterile tubes were prepared and labeled sequentially. To each tube, DMEM media (0.9 ml) was added. The FMDV stock was thawed at 37°C in water bath, and virus stock (0.1 ml) was added to first tube. The mixture was thoroughly mixed by using pipette. A 10-fold serial dilution was then performed by transferring 0.1 ml of the virus suspension from the first tube to the second, and continuing this dilution process to the fifth tube, achieving final dilutions of 10^{-1} to 10^{-5}. From each of the tubes, virus dilution 100 µl to each wells was then added, with four replicates per dilution (rows 1-5), while row 6 served as an uninfected control. The plates were incubated at 37°C for 48-72 h in a CO_2 incubator. During the incubation, the cells in flasks were regularly monitored under inverted microscope for CPE in cells. The number of wells showing CPE (positive) or not showing CPE (negative) was recorded. The virus dilution that produced the highest $TCID_{50}$ values was selected for subsequent studies. Cells showing 50% and 75% CPE were assigned (+) and (++) score. Negative score (-) was given for cells showing no CPE and if the monolayer of the cells were totally destroyed a score of (+++) was assigned.

The $TCID_{50}$ was determined using Karber's method (Burleson *et al*., 1992) according to the formula given below:

$$Log_{10}\ TCID_{50} = L - d\ (s - 0.5)$$

where,

L = Log_{10} of the dilution with the highest concentration of the virus.

d = Logarithmic dilution factor

s = The sum of proportions

2.5 Virus inhibition assay (IC_{50})

The antiviral effectiveness of phytochemicals against FMDV in BHK-21 cells was evaluated using the MTT assay, 3-(4,5-di-methyl-thiazol-2-yl)-2,5-di-phenyl-tetrazolium bromide. The procedure involved dissolving a pre-measured amount of MTT reagent in assay buffer, then adding it to the cell culture. To prepare the MTT reagent, 6 ml of cell-based assay buffer was aseptically added to a vial of MTT, which was thoroughly dissolved by vortexing and the resulting solution had a concentration of 5 µg/ml. The reconstituted reagent was then filtered in sterilized syringe filter (0.22 µm) and stored in amber bottle at -20°C until further use. BHK-21 cells were then seeded into 25 cm^2 flasks and incubated (37°C) in a 5% CO_2 incubator for 24 h until they reached confluency. The cells were subsequently harvested and 100 µl solution, containing 1×10^3 cells per well, were placed into 96-well plate in triplicate, with subsequent incubation (37°C) for 24 h. Control wells contained only BHK-21 cells with maintenance media. Phytochemicals were added to the wells at concentrations below their respective CC_{50} values, ranging from 1.95 to 250 µg/ml, using two-fold dilutions. Incubation of plates was done for 2 to 4 h at 37°C with 5% CO_2. After that, FMDV at $10^{6.25}$ $TCID_{50}$ to each well was added and plates were incubated for 48 h. Virus control wells received only FMDV in maintenance media. Subsequent to incubation, the medium was removed from each well and substituted with 0.5% MTT solution (100 µl). Then plates were incubated for additional 4 h at 37°C. After adding 10% DMSO (100 µl) to each well, MTT solution was discarded and incubated for 2 h (37°C). The OD (optical density) of wells were measured at 570 nm to assess cell viability.

$$\% \text{ Antiviral activity} = \frac{\text{O.D. (Test)} - \text{O.D. (Virus Control)}}{\text{O.D. (Cell Control)} - \text{O.D. (Virus Control)}} \times 100$$

2.6 Selectivity index (SI)

The selectivity index was determined as the ratio of the CC_{50} to IC_{50} of the virus.

2.7 Statistical analysis

All the procedures were carried out in triplicate. The *p*-value of < 0.05 was referred as significant using ANOVA. CC_{50} and IC_{50} were determined through linear regression, based on intercept of the slope of best fit line.

3. Results

3.1 Cytotoxicity assay in BHK-21 cells (CC_{50})

The cytotoxicity of phytochemicals in BHK-21 cells were assessed using the dye exclusion technique with trypan blue (Figure 1). The CC_{50} was calculated using a linear regression equation based on the percentage of living cells across the phytochemical dilution range of 1.95 to 1000 µg/ml. Rounded cells, granulations, intracellular matrix loss, and cell detachment were noted in the BHK-21 cell lines as a cytopathic effect (Figure 2). The result of the cytotoxicity (CC_{50}) of plant phytochemicals in the BHK-21 cells is presented in the table below (Table 1), and the linner regression graph of the CC_{50} of curcumin, eugenol, and azadirachtin in BHK-21 cell line is presented in Figures 3-5.

Table 1: Cytotoxic concentration-50% (CC_{50}) value of plant phytochemicals in BHK-21 cell line

Phytochemicals	CC_{50} (Mean ± S.E.)
Curcumin	285.79 ± 3.02 µg/ml
Eugenol	204.53 ± 1.22 µg/ml
Azadirachtin	129.37 ± 1.04 µg/ml

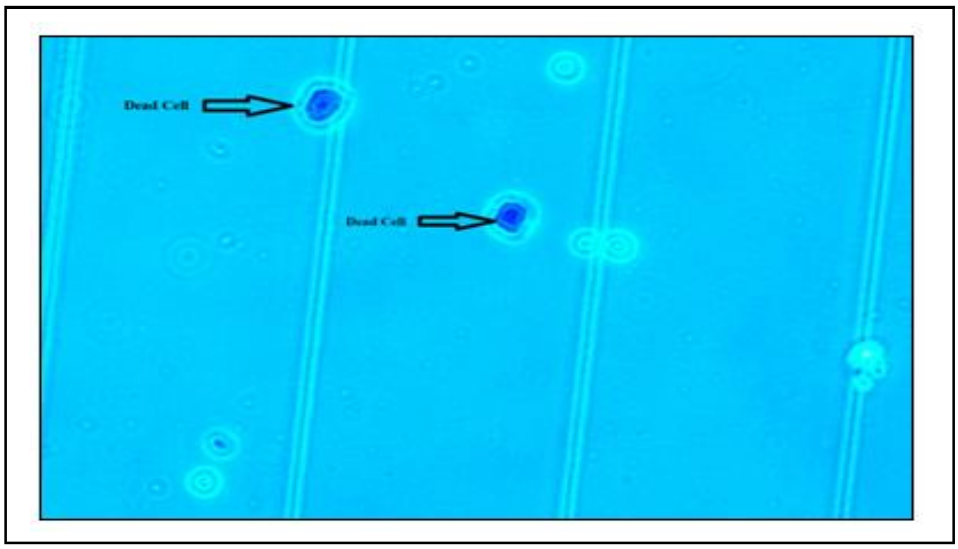

Figure 1: Viable (unstained) and non-viable (stained) BHK-21 cells in trypan blue staining.

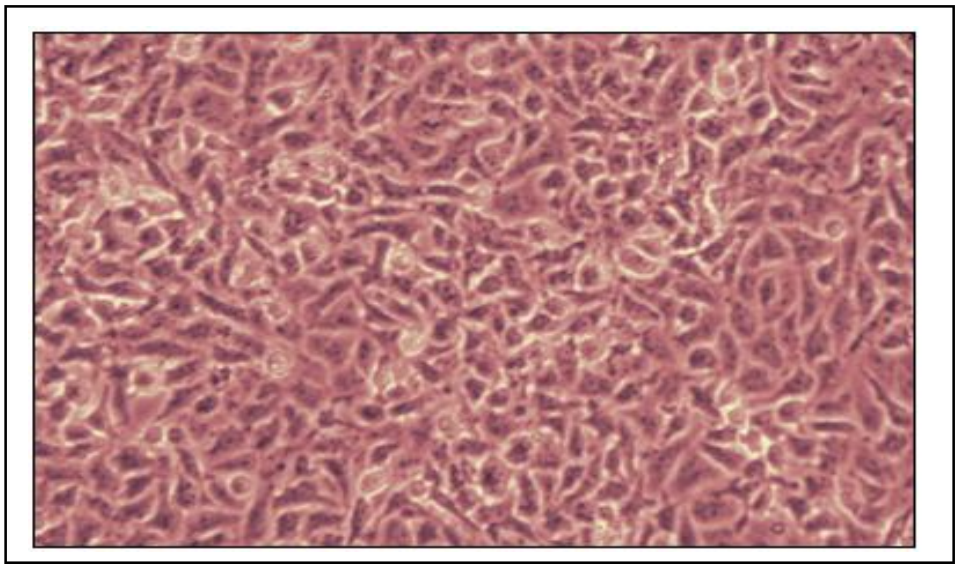

Figure 2: Representative photograph showing CPE in BHK-21 cells infected with FMD virus, 24 h post infection.

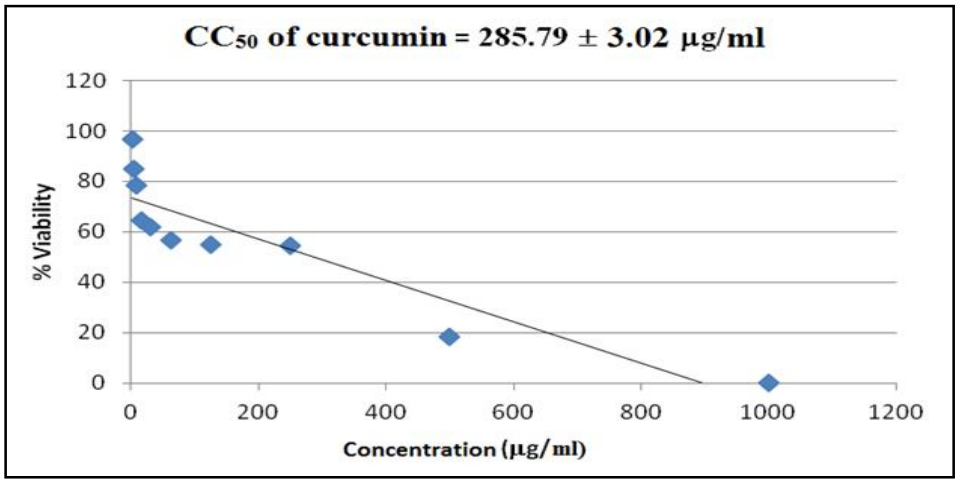

Figure 3: Linear regression plot of CC_{50} of curcumin in BHK-21 cells.

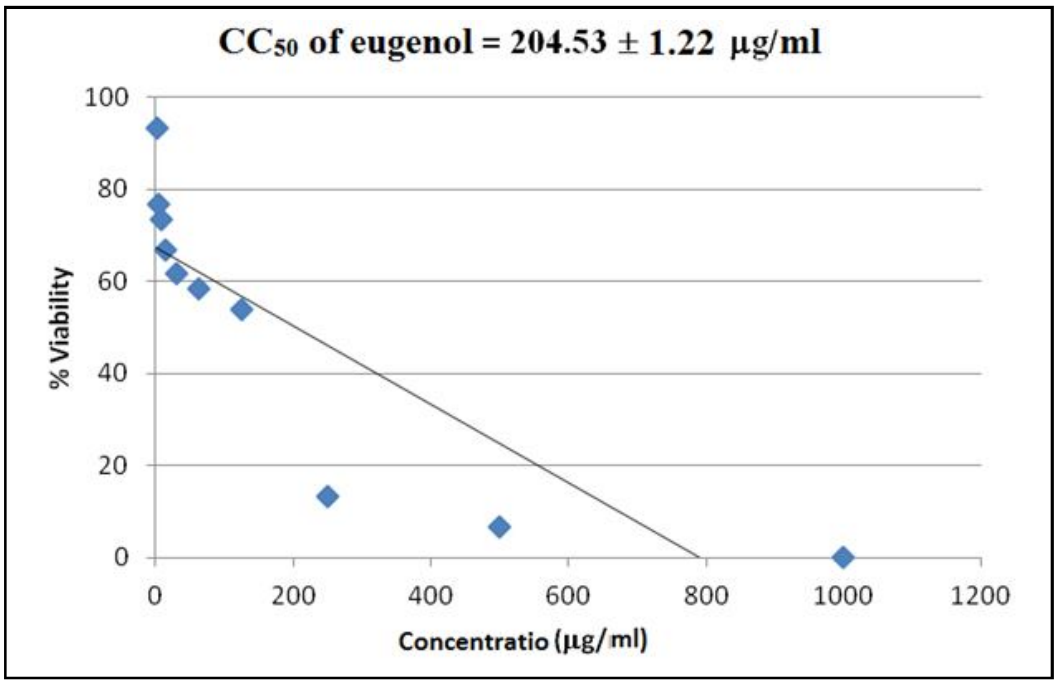

Figure 4: Linear regression plot of CC_{50} of eugenol in BHK-21 cells.

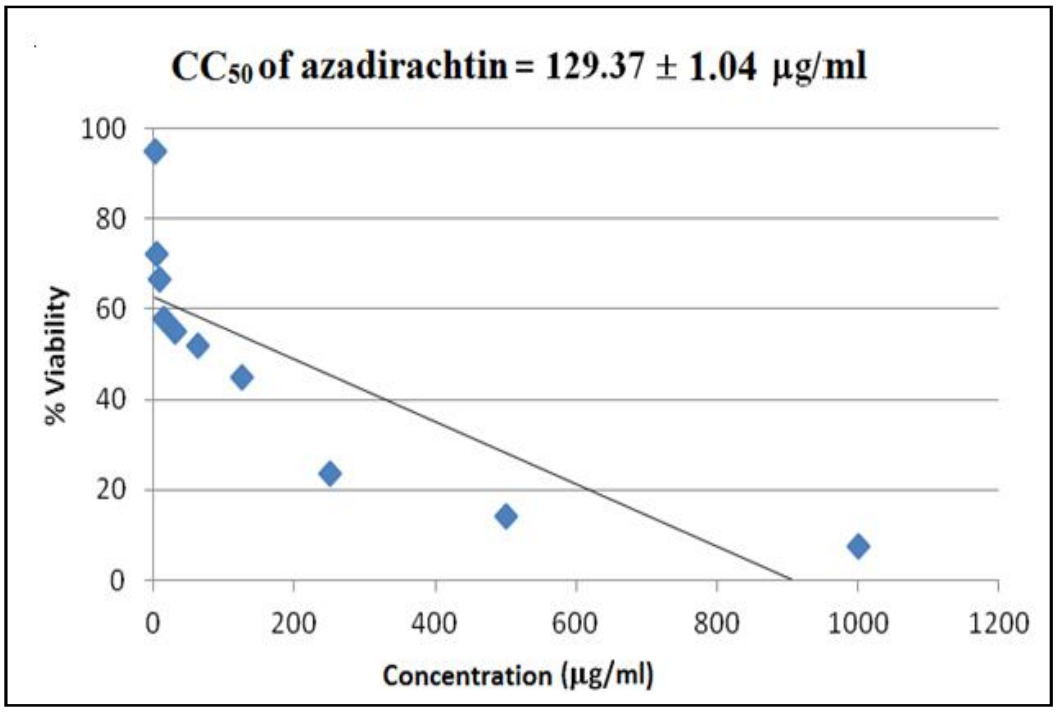

Figure 5: Linear regression plot of CC_{50} of azadirachtin in BHK-21 cells.

3.2 Virus inhibition assay (IC_{50})

The IC_{50} values against the FMD virus were determined by using the online software tool ED-50-V10 (Readme); an Excel add-in was utilized to compute IC_{50} values. The IC_{50} values of various treatment groups were presented in Table 2, and the linear regression graph of the IC_{50} of curcumin, eugenol and azadirachtin in the BHK-21 cell line is presented in Figures 6-8.

Table 2: Virus inhibition concentration – 50% (IC_{50}) value of plant phytochemicals in BHK-21 cell line

Phytochemicals	Virus inhibition –50% (IC_{50})
Curcumin	25.53 ± 1.48 µg/ml
Eugenol	25.57 ± 1.25 µg/ml
Azadirachtin	39.37 ± 0.10 µg/ml

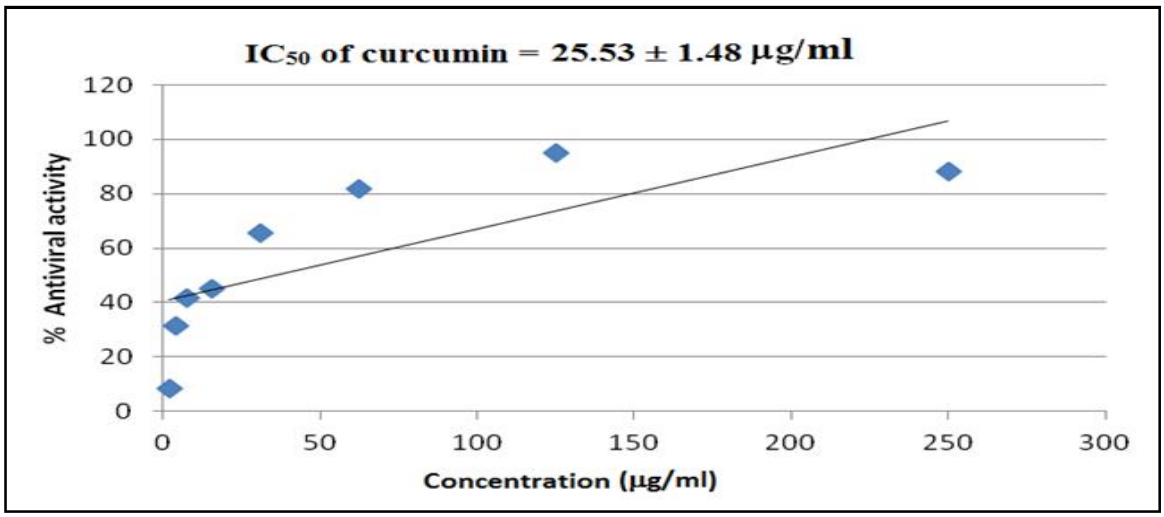

Figure 6: Linear regression graph displaying the IC_{50} following treatment with curcumin in BHK-21 cell line.

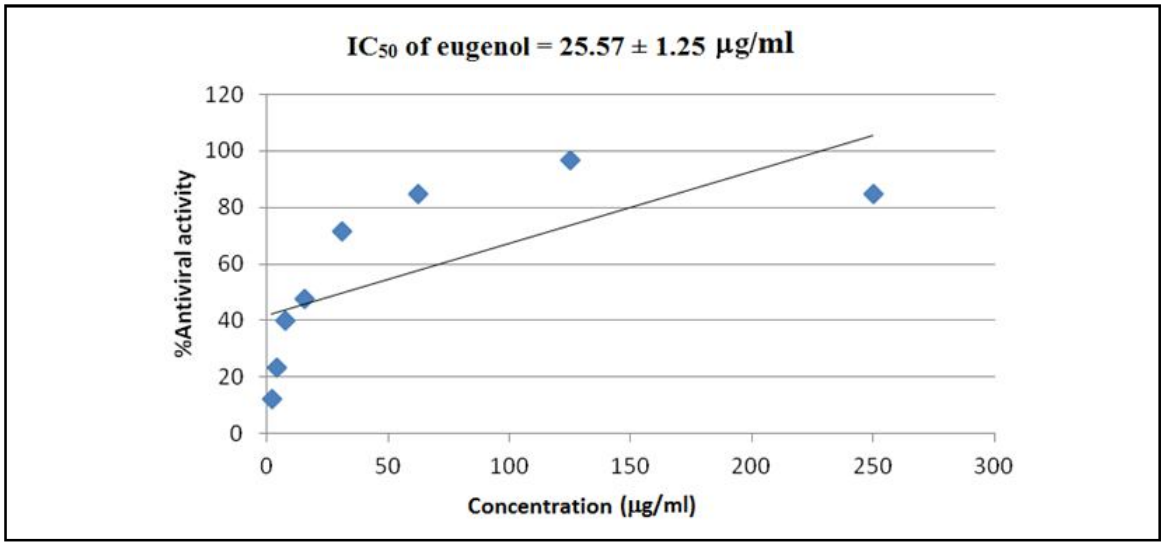

Figure 7: Linear regression graph displaying the IC_{50} following treatment with eugenol in BHK-21 cell line.

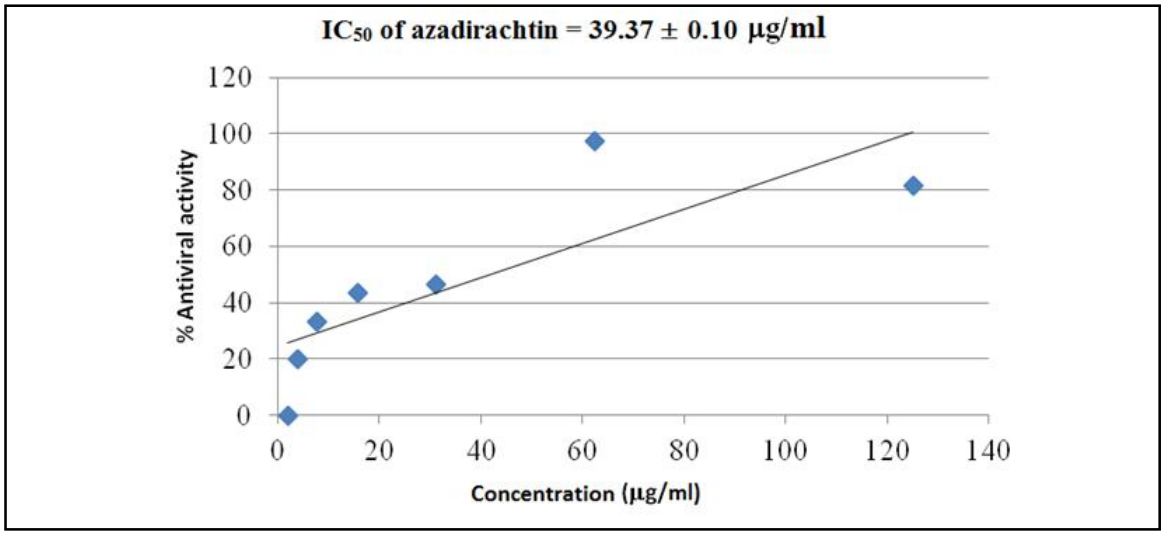

Figure 8: Linear regression graph displaying the IC_{50} following treatment with azadirachtin in BHK-21 cell line.

3.3 Selectivity index (SI)

The SI of curcumin, eugenol, and azadirachtin were found to be 11.22 ± 0.60, 8.07 ± 0.71, and 3.27 ± 0.04, respectively.

4. Discussion

4.1 Tissue culture infectious dose-50% ($TCID_{50}$)

In this study, the infected FMDV (serotype O) titrated in BHK-21 (10^{th} passages) had an average log tissue culture infective dose-50 (Log_{10} $TCID_{50}$) of 6.252 ± 0.022 ($10^{6.25}$ $TCID_{50}$). Similar $TCID_{50}$ values for FMDV titrated in BHK-21 cell culture were reported also by Imran *et al*. (2016), Saher *et al*. (2018) and Younus *et al*. (2017) and the values ranged from 10^6 to $10^{6.37}$. The infectivity of the virus and the particular type of cell line used can have an effect on the $TCID_{50}$ value.

4.2 Cell cytotoxicity studies (CC_{50})

In this study, the CC_{50} value of curcumin in the BHK-21 cell line was estimated as 285.79 ± 3.02 µg/ml. In another investigation, Zandi *et al*. (2010), El-Toumy *et al*. (2018) and Namitha, (2019) reported that the CC_{50} value of curcumin in the "Vero" cell line was 484.20 µg/ml, 49.8 ± 0.40 µg/ml, and 290.40 ± 1.419 µg/ml, respectively. The average CC_{50} for eugenol in the BHK-21 cells was estimated in this study was 204.53 ± 1.22 µg/ml. Namitha, (2019) reported CC_{50} value to be 319.70 ± 1.301 µg/ml for curcumin in "Vero" cells. Padilla *et al.* (2013) determined the CC_{50} of 29.5 mM of curcumin in BHK-21 cells infected with dengue virus type-2 (DEN-2). The estimated CC_{50} for azadirachtin in this study was 129.37 ± 1.04 µg/ml. In another research done by Parida and colleagues (2002), it was found that azadirachtin did not have any inhibitory effect against DEN-2 virus replication in both *in vitro* as well as *in vivo*. Parvez *et al*. (2019) reported that azadirachtin did not show cytotoxicity in "human hepatoblastoma" cell line even at concentration of 50 µg/ml. The CC_{50} values observed in various studies may differ because of the differences in cell line types utilized, the conditions under which they are cultured, and other related laboratory procedures employed.

4.3 Virus inhibition studies (IC_{50})

The current study determined that the average IC_{50} value of curcumin was 25.53 ± 1.48 µg/ml. Zandi *et al*. (2010) used the "Vero" cells to assess the antiviral qualities of curcumin-derivatives against "herpes simplex virus type-1" (HSV-1). The results revealed that curcumin and curcumin-derivatives have strong antiviral activity against HSV-1 in "Vero" cell line. Padilla *et al.* (2013) estimated the IC_{50} of 11.51 mM of curcumin for "dengue virus type-2" (DEN-2) in BHK-21 cells. Khosropanah *et al*. (2016) found that curcumin's IC_{50} value in "MDA-MB-231 cell line" ranged from 30.78 to 79.58 µg/ml and 33 µg/ml in "Vero" cells. The average IC_{50} of eugenol estimated in the present study was 25.57 ± 1.25 µg/ml. Benencia and Courrèges (2000) reported the IC_{50} value of eugenol against "herpes Simplex virus" (HSV-1 and HSV-2) as 16.2 µg/ml and 250 µg/ml, respectively. In the current study, average IC_{50} of azadirachtin was found to be 39.37 ± 0.10 µg/ml. Parvez *et al*. (2019) reported more than 52.5% inhibitory effect of azadirachtin against "hepatitis B virus" (HBV) compared to control. Differences in IC_{50} values across various studies could be due to distinct types of viruses employed, infectivity of the virus types, passage numbers, cell lines utilized, and other cultural conditions.

4.4 Selectivity index (SI)

The average selectivity index of plant phytochemicals used in this study was found to be 3.27 ± 0.04, 8.07 ± 0.71, and 11.22 ± 0.60, respectively. Padilla *et al.* (2013) reported a selectivity score of 2.56 of curcumin for "dengue virus" in the BHK-21 cells. In the study carried out by Zandi *et al*. (2010) the SI values of gallium-curcumin, curcumin and Cu-curcumin were 18.4, 14.6 and 14.1, respectively. The selectivity index is useful for identifying substances that are suitable for further development and to assess the effectiveness as well as safety of a product (Ichsyani *et al*., 2017). A high SI indicates theoretically more effective and safer compound for *in vivo* treatment against a given viral infection. The reported SI values in different studies vary due to several factors like, kind of virus used, type of plant extracts, method of extraction, cell line used, and cell culture conditions.

5. Conclusion

The CC_{50} value showed a significant difference among the phytochemicals studied. Azadirachtin (129.37 ± 1.04 µg/ml) showed relatively higher cytotoxicity in BHK-21 cells compared to curcumin (285.79 ± 3.02 µg/ml) and eugenol (204.53 ± 1.22 µg/ml). The IC_{50} values of phytochemicals revealed that, curcumin (25.53 ± 1.48 µg/ml) and eugenol (25.57 ± 1.25 µg/ml) showed better virus inhibitory activity than azadirachtin (39.37 ± 0.10 µg/ml). While IC_{50} values of the phytochemicals differed significantly from each other. Azadirachtin showed narrower selectivity with SI value of 3.27 ± 0.04 than curcumin (11.22 ± 0.60) and eugenol (8.07 ± 0.71). The plant phytochemicals showed promising results that suggest the possibility of using them as potential antiviral agents against FMDV. While this study revealed some key observations into the *in vitro* antiviral activity of curcumin, eugenol, and azadirachtin against FMDV, there is potential for future research such as, conducting *in vivo* in animal models studies to validate the antiviral efficacy of the compounds, characterizing the compounds in more detail, using multiple cell lines, investigating the molecular mechanisms, and comparing the efficacy with existing antiviral therapies.

Acknowledgements

The author expresses heartfelt thanks to the faculty and staff at the CVSc, AAU, Khanapara-781022, Guwahati, Assam, India and LCVSc, AAU, Joyhing-787051, North Lakhimpur, Assam, India, for their contribution and support for successful completion of the research.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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Citation **Himangshu Baruah, Jadav Sarma, Sanjib Khargharia, Snigdha Hazarika, Aditya Baruah and Manoj Kumar Kalita (2024). *In vitro* antiviral and cytotoxicity assessment of curcumin, eugenol and azadirachtin in foot and mouth diseases virus in BHK-21 cells . Ann. Phytomed., 13(2):484-490. http://dx.doi.org/10.54085/ap.2024.13.2.48.**