IN VITRO ANTIVIRAL ACTIVITY OF STEM AND LEAF EXTRACT OF *OLEA FERRUGINEA ROYLE* AGAINST FOOT AND MOUTH DISEASE VIRUS

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ABSTRACT: The present study was done to discover the natural agent of antiviral that could be essentially and simply utilized in the field, to lessen the animal's carries condition or to decrease the infection in animals during the outbreak of disease. Culture technique was used to analyze the antiviral or cytotoxic effect of *Olea ferruginea* Royle against FMD virus. Four kinds of extracts of leaf and stem in polar and non-polar solvents (n-hexane, alcohol, aqueous and chloroform) were used. The extract of n-hexane stem showed toxicity at concentration range of 31.25 µg/mL-125 µg/mL while showed cytotoxicity at 3.9µg/mL to 125µg/mL along with extract showed CSP 54% at 62.5µg/mL or 58% CSP at 125µg/mL concentration, respectively. Cytotoxicity potential of chloroform extract was measured at 500 to 2000 µg/mL which showed that the extract was virucidal at 125µg/mL concentration against FMDV. Likewise, the antiviral significant potential was documented by *Olea ferruginea* Royle's alcohol leaf extract at 15.62µg/mL-250µg/mL concentration with 52% to 59% CSP, while the cytotoxic activity of leaf extract was observed at 1000 µg/mL to 2000µg/mL. In the same way, the aqueous leaf extract of *Olea ferruginea* Royle was observed as virucidal at 62.5µg/mL concentration. In short, *Olea ferruginea* Royle was used against FMDV due to its antiviral potential.

Keywords: *Olea ferruginea*, Cytotoxicity, Antiviral activity, MTT Assay, BHK-21 cells, FMDV

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INTRODUCTION

***Olea ferruginea* Royle:** *Olea ferruginea* Royle commonly known as Indian and Kao olive, is occupied in bushes or trees or has shading as grayish-green. Its youthful bark turns into thin strips when peeling off at developing old. Its upper surface is sparkling and dull green, cuspidate regularly along with moment scales as a thick layer along with established leaves. The trees drupe 0.8mm long, width is 5mm, oil, mash meager or dark on ripening (Amin *et al*., 2013). It is used to relieve throat ache, tooth and strengthen the gums with a decoction of fresh leaves (Masood *et al*. 2019). In addition, olive also contains various compounds as bioactive that have the potential of antihypertensive, antioxidant, anti-bacterial and anti-inflammatory (Upadhyay *et al.,* 2010; Aliabadi *et al.,* 2012; Lucas *et al.,*2011; Nora *et al*. 2012). Imadi *et al.,* (2018) explained that plants were used as a source of medicine by people of rural or tribal areas with the help of experienced and generation of knowledge. Globally, almost 500,000 plant species exist in which some are used as an agent for antiviral activity due to the presence of many phytochemicals in them like alkaloids, flavonoids and phenolic which exhibit the potency of antiviral (Meenakshi *et al.,* 2020).

Foot and mouth disease of animals occurred by a virus which is called as FMDV. It is a highly infectious disease of wild animals, cattle, pigs, goats, and sheep. The virus that is FMD virus becomes a causal agent for this disease which influences domesticated animals and livestock; causes an intense sickness that is characterized by fever, vesicular, feet' sores, nose tongue or teals with low morality and high dismalness. This causative agent has seven types of serotypes, so it is varied genetically and vaccination of one serotype does not work against another serotype's infection. Antiviral activity of plant in the estimation of optical density (O/D) was documented that agreed with study of Tewari *et al*.(2020), who explained that when cells were stained by using crystal violet or methylene blue then the living cells got stain and look like blue. The optical density of these cells was calculated after de-staining by utilizing alcohol. MTT assay was used to calculate the viability of cells. In such tests, activity depends upon mitochondrial dehydrogenase which changes the MTT salt into violet formazan that was water-soluble in living cells. Formazan has quantity according to the quality of living cells. Such investigation relates to the findings of Srivastava *et al.,* (2016) who documented that the assay of MTT was reliable and fast. In the present study, cytotoxicity was checked with the

help of plant extract for BHK-21 cells. Several observations were done to analyze the cytotoxicity of plant extract by using BHK-21 cells (Nugraha *et al*. 2015; Younus *et al*. 2015). Similarly, against the virus of Japanese Encephalitis the antiviral potential of *Isatis indigotica* was checked along with BHK-21 cells were also used to check the extract toxicity. Chang *et al*.,(2012) explained in their study that generally actions of Phyto-antiviral agents were exerted by interfering with synthesis of amino acid which was essential for inactivating the viruses as well as represented the assembly or shedding the virus at the cell membrane and stop directly infected cells, penetrating or stop the replication of the virus. The present study was done to discover the natural agent of antiviral that could be essentially and simply utilized in the field to lessen the animal's carries condition or to decrease the infection in animals during the outbreak of disease. Culture technique used to analyze the antiviral or cytotoxic effect of *Olea ferruginea* Royle against FMD virus, four kinds of extracts of leaf and stem of in polar and non-polar solvents were used in this study. The objective of this study was to find out ethnopharmacological effect of *Olea ferruginea* Royle against FMDV as this virus is the causal agent of foot and mouth disease of livestock animals.

Fig 1 *Olea ferruginea* Royle

MATERIALS AND METHODS

2.1. Instruments: Syringes 5mL and 10 mL, Glass filtration assembly, 96-well cell culture plates, Petri dishes, ELISA, Centrifuge, and hemocytometer.

2.2 Chemicals: These include 0.25% trypsin solution, Disodium hydrogen phosphate, Fetal bovine serum, dNTPs, Bicarbonate/ Carbonate buffer, Cell culture media M-199 Chloroform, n-hexane, alcohol, DMSO, Trypan blue, sodium chloride, and MTT reagent.

2.3 Stock solution's preparation: Ethanolic, aqueous, and hexane used its 0.02g for the preparation of stock solution that was extracted from dried parts of the plants, and then these were suspended in 1 mL of cell culture media that termed as maintenance media. However, 1 mL of 1 % DMSO suspended the 0.02g of each extracted that came from chloroform dried parts of plants to prepare a 20,000X stock solution. Then, in a cabinet that was safety stock solutions with 0.22 µm syringe filters were filtered.

2.4 Extracts Dilutions: In this study, 2000µg/mL, 1000µg/mL, 250µg/mL, 500µg/mL, 125µg/mL, 62.5µg/mL, 31.25µg/mL, 7.8 µg/mL, 3.9 µg/mL and 15.62 µg/mL were considered as desired concentrations for each plants' part. The dilutions at required range of concentration were prepared at twice.

2.5 cell culture media preparation: 1.2g of the powdered M-199 was included in 100 mL of two-fold refined water along with fetal bovine serum (1% fetal bovine serum for upkeep media that was maintenance media, antibiotics, and 10% serum for growth media) according to Greham (1993). Then, the media with the help of filtration assembly that had negative pressure in a safety cabinet was filtered.

2.6 Cell Line: From WTO-QDL of the University of Veterinary and Animal Sciences, Lahore, Pakistan, a cell line of BHK-21 cell was obtained and through a hemocytometer, the quantification of dead as well as viable cells was done.

2.7 Quantification of BHK-21 cells: For the quantification of cells in this study, a cleaned hemocytometer was used. Sample for this was prepared by mixing a drop that was 0.4 percent. In the hemocytometer's counting chamber, a drop of suspension along with trypan blue was loaded and then it was placed on a microscope to count viable (unstained) and dead (stained) cells which were quantified by microscope.
To calculate the percentage of viable cell the following formula was used:

$$\% \text{ Viable cells} = \frac{\text{Number of viable cells / mL}}{\text{Total number of cells / mL}} * 100$$

2.8 Virus Stock: FMD virus was acquired from WTO-Quality Operation Laboratory (QOL), the University of Veterinary and Animal Sciences, Lahore. Its $TCID_{50}$ was calculated after Reed and Muench, 1938 (Cavalcante *et al*. 2020).

2.9 Virus Inoculation protocol: In a flask of cell culture, the virus was inoculated by a monolayer of BHK-21 cells. Growth development media was evacuated from the flask that containing a blended monolayer of BHK-21 cells and that monolayer was rinsed twice with PBS. With the help of a syringe filter that was 0.22 µm, the FMDV in cell culture media 250 µl was filtered and mixed along with it distributed evenly on the monolayer. For cytopathic effects (CPE) eighty to nighty percent (80-90%) of the inoculated cells were observed under an inverted microscope regularly while CPE was observed on the 6th day of incubation.

2.10 Virus harvesting: Overnight, at -20ºC the infected flask was kept after that at room temperature the flask was thawed and three times this procedure was repeated. Then, the viral suspension was transmitted to the appendorf tube, after that the suspension at 5000 rpm was centrifuged at 4ºC for 5 to10 min. This method separates a cell's pellet of debris at the bottom. After that, the supernatant was stored at a temperature of -70ºC until it was used.

2.11 Tissue culture infective dose 50 (TCID50): Virus suspension's dilution as serial of tenfold was prepared in the maintenance of cell culture that was from 10^1 to 10^{10}. A plate of 96 well-cell cultures was taken containing a BHK-21's blended monolayer of cells that removing the growth media from each well. In the first column, each well was poured with 100µl dilution of a virus, while the last two wells that contained maintenance media and cells of each plate were used as control. Then, under the inverted microscope, each plate at 37ºC in the incubator was kept with 5% of CO_2, the CPE of the virus was examined two times daily. Wells that were infected by the virus were compared with the wells that controls the cells and then marked either they were positive or negative (Reed and Muench, 1938).

The following formula was used to calculate the TCID50:

$$\text{P.D. (Proportionate Distance)} = \frac{\text{Percentage infectivity above 50\% - 50\%}}{\text{Percentage infectivity above 50\% - Percentage infectivity below 50\%}}$$

RESULTS

To study the effect of phytochemicals in cell culture, extracts were checked by using different cytotoxic assays and types of cell lines (Kim, 2018). For the evaluation of the cytotoxic potential of a compound, MTT assay is widely used. It is used to measure the reducing potential of the cell. MTT reagent with the help of viable cells will be reduced to formazan that was a colored product (Azeem *et al*. 2015). Through MTT assay, moderate cytotoxicity along with the antiviral activity of *Syringa* plants of Oleaceae against human cancer cells was observed by Su *et al.,* (2015). While, cytotoxicity of *Ficus deltoidea* on ovarian human carcinoma cells was checked by using MTT assay of standard coulometric (Akhir *et al*. 2011).

3.1 *Olea ferruginea* n-hexane stem extract: For BHK-21 cells, the cytotoxic analysis was performed to observe n-hexane stem extract of *Olea ferruginea* which exhibit various values of CSP which their respective concentration range such as 80% at 3.9µg, 77% at 7.8µg, 71% at 15.62µg/mL, 65% at 31.25µg/mL 62% at 62.5µg/mL, 54% at 125µg/mL, 42% at 250µg/mL, 35% at 500µg/mL, 25% at 1000µg/mL and 10% at 2000µg/mL (Table 1), it was noted that 3.9µg/mL-125µg/mL concentration have more than 50% CSP, so; their extract was observed as non-cytotoxic. Meanwhile, CSP that was less than fifty percent (50%) was experienced 250µg/mL-2000µg/mL concentration range and gave activity of cytotoxicity. On the other hand, the activity of antiviral against FMDV of n-hexane *Olea ferruginea* leaf extract was documented at 125µg/mL concentration with 53% CSP (Table 2, Fig 2). If the concentration range of extract was low such as 3.9µg/mL-62.5µg/mL as well as the high range of concentration like 250µg/mL-2000µg/mL with less than 50% CSP showed no activity of antiviral.

Table 1: Cytotoxic activity of *Olea ferruginea* stem *n*-hexane extract for BHK-21 cells.

Sr. No.	Conc. used (µg/mL)	Mean O.D. value	Cell survival percentage (%)
1	3.9	0.503±0.05a	80
2	7.8	0.487±0.03b	77
3	15.62	0.455±0.02c	71
4	31.25	0.421±0.02d	65
5	62.5	0.408±0.05e	62
6	125	0.366±0.04f	54
7	250	0.302±0.05g	42
8	500	0.267±0.03h	35
9	1000	0.216±0.05i	25
10	2000	0.137±0.01j	10

Table 2: Antiviral Activity of *Olea ferruginea* stem *n*-hexane extract against FMDV

Sr. No.	Conc. used (µg/mL)	Mean O.D. value	Cell survival percentage (%)
1	3.9	0.184±0.01i	19
2	7.8	0.215±0.03h	25
3	15.62	0.256±0.02f	33
4	31.25	0.278±0.02e	37
5	62.5	0.329±0.01b	47
6	125	0.358±0.04a	53
7	250	0.391±0.01c	41
8	500	0.287±0.03d	39
9	1000	0.264±0.02ef	35
10	2000	0.222±0.04g	27

*Different alphabets in the columns indicate the significant difference at 0.05 significant level
* S.D.= Standard Deviation

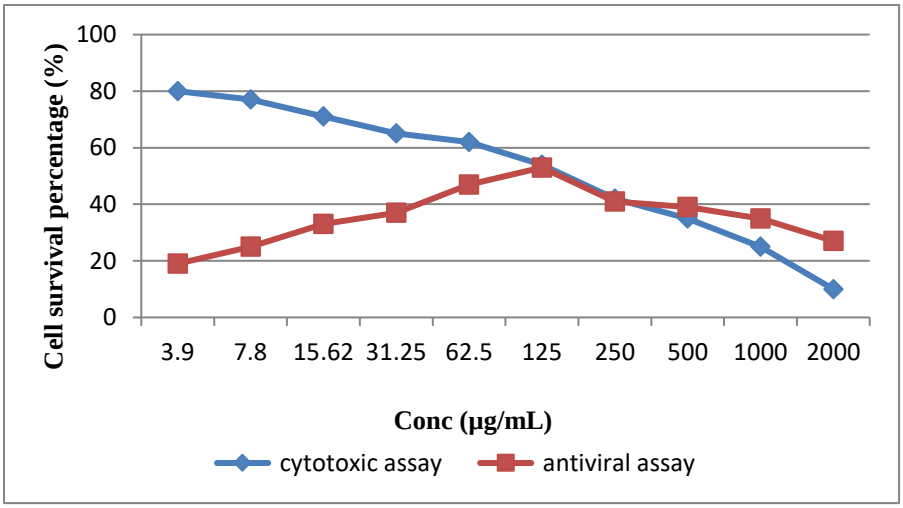

Fig 2: Comparison between antiviral and cytotoxic activity of *n*-hexane *Olea ferruginea* stem extract

***3.2 n*-hexane *Olea ferruginea* leaf extract:** Secondly, cytotoxicity for n-hexane leaf extract of *Olea ferruginea* was observed at various concentration against BHK-21 cells. The concentration and their respective CSP were 3.9µg at 79%, 7.8µg at 76%, 15.62µg/mL at 69%, 31.25µg/mL at 63%, 62.5µg/mL at 56%, 125µg/mL at 50%, 250µg/mL at 44%, 500µg/mL at 42%, 1000µg/mL at 39% and 2000µg/mL at 35% (Table 3, Fig 3). The range of concentration which have more than fifty percent (50%) CSP was considered as non-cytotoxic and that was 3.9µg/mL-25µg/ mL, while on the other hand, 250µg/ mL-2000µg/ mL concentration have less than fifty percent (50%) CSP and documented as toxic against BHK-21 cells. Likewise, antiviral activity of leaf extract have fifty four percent (54%) and fifty eight percent (58%) CSP at 62.5µg/mL or 125µg/mL concentration

respectively which showed antiviral activity because CSP observed more than fifty percent (50%). While, at 250µg/mL-2000µg/mL range did not show any antiviral activity because this range have CSP less than fifty percent (50%) (Table 4). The concentration range of non-cytotoxic was also non-virucidal that is 3.9-31.25µg/mL.

Table 3: For BHK-21 cells the cytotoxic activity of *Olea ferruginea n*-hexane leaf extract

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.495±0.04a	79
2	7.8	0.481±0.01b	76
3	15.62	0.445±0.02c	69
4	31.25	0.415±0.01d	63
5	62.5	0.376±0.01e	56
6	125	0.345±0.03f	50
7	250	0.315±0.04g	44
8	500	0.301±0.03h	42
9	1000	0.288±0.03i	39
10	2000	0.267±0.05j	35

Table 4: against FMDV the antiviral activity of *Olea ferruginea* leaf *n*-hexane extract.

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.193±0.03h	21
2	7.8	0.227±0.02g	28
3	15.62	0.274±0.04f	37
4	31.25	0.339±0.01c	49
5	62.5	0.368±0.02b	54
6	125	0.387±0.02a	58
7	250	0.325±0.04d	46
8	500	0.391±0.05e	41
9	1000	0.274±0.01f	37
10	2000	0.235±0.03g	29

* at 0.05 significant level the significant difference indicated by different alphabets in the columns
* S.D means that Standard Deviation

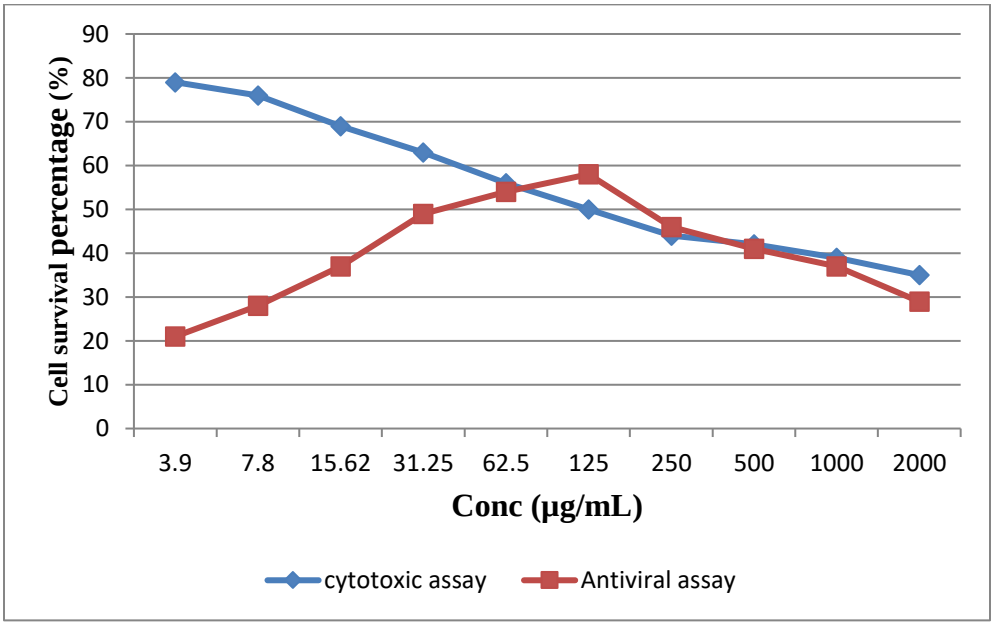

Figure 3: Comparison between the cytotoxic and antiviral activity of *Olea ferruginea n*-hexane leaf extract.

***3.3 Olea ferruginea* chloroform stem extract:** After the cytotoxic analysis of leaf n-hexane extract, the cytotoxic activity of chloroform stem *Olea ferruginea* extract was observed for BHK-21 cells. In which the concentration range was observed with their respective CSP and that were 3.9µg/mL at 78%, 7.8µg/mL at 73%, 15.62µg/mL at 69%, 31.25µg/mL at 66%, 62.5µg/mL at 61%, 125µg/mL at 58%, 250µg/mL at 54%, 500µg/mL at 49%, 1000µg/mL at 45% and 2000µg/mL at 41% (Table 5, Fig 4). At 3.9µg/mL-250µg/mL, the extract concentration was experienced non-toxic against BHK-21 cells because it has more than fifty percent (50%) CSP. Meanwhile, range from 500µg/mL-2000µg/mL was cytotoxic against BHK-21 cells because its CSP was observed less than (50%) fifty percent. When antiviral activity for chloroform stem extract of *Olea ferruginea* against FMDV was measured at above mention concentration then that extract was documented antiviral at 250µg/mL concentration range with 57% CSP (Table 6). While ranges of lower concentration that were 3.9µg/mL-125µg/mL have characteristics of non-cytotoxic for BHK-21 cells but did not possess any antiviral activity at this concentration range. Likewise, the extract was observed non-virucidal at an increased range of concentrations that are 500µg/mL-2000µg/mL.

Table 5: Cytotoxic activity of *Olea ferruginea* stem chloroform extract for BHK-21 cells

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.489±0.02a	78
2	7.8	0.467±0.01b	73
3	15.62	0.444±0.03c	69
4	31.25	0.429±0.01d	66
5	62.5	0.402±0.04e	61
6	125	0.384±0.02f	58
7	250	0.367±0.03g	54
8	500	0.341±0.02h	49
9	1000	0.317±0.02i	45
10	2000	0.392±0.03j	41

Table 6: Against FMDV the antiviral activity of *Olea ferruginea* stem chloroform extract

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.173±0.04h	17
2	7.8	0.199±0.02g	22
3	15.62	0.217±0.02f	26
4	31.25	0.273±0.04e	36
5	62.5	0.296±0.05d	41
6	125	0.339±0.01b	49
7	250	0.379±0.02a	57
8	500	0.311±0.04c	44
9	1000	0.278±0.02e	37
10	2000	0.225±0.01f	27

* At 0.05 significant level, the significant difference was indicated by different alphabets in the columns
* S.D means that Standard Deviation

***3.4 Olea ferruginea* chloroform leaf extract:** In addition, cytotoxic activity was measured against BHK-21 cells of chloroform *Olea ferruginea* leaf extract. In which different concentrations was documented with their relative CSP such as 2000µg/mL, 1000µg/mL, 500µg/mL, 250µg/mL, 125µg/mL, 62.5µg/mL, 31.25µg/mL, 15.62µg/mL, 7.8µg/mL, 3.9µgmL. In these 3.9µgmL-500µg/mL, the range of concentration has more than fifty percent (50%) CSP due to which it showed non-cytotoxic effect (Table 7). While 1000µg/mL-2000µg/mL, range of concentration observed as cytotoxic because it had less than fifty percent (50%) CSP. Meanwhile, antiviral activity of chloroform leaf extract was also examined which observed at 31.25µg/mL, 62.5µg/mL or 125µg/mL concentration range with 50%, 52% and 55% CSP respectively against FMDV (Table 8, Fig 6). The non-virucidal potential of the extract was observed at a low range of concentrations that are 3.9µg/mL-15.62µg/mL, as well as 250µg/mL-2000µg/mL, increased range with less than fifty percent (50%) CSP.

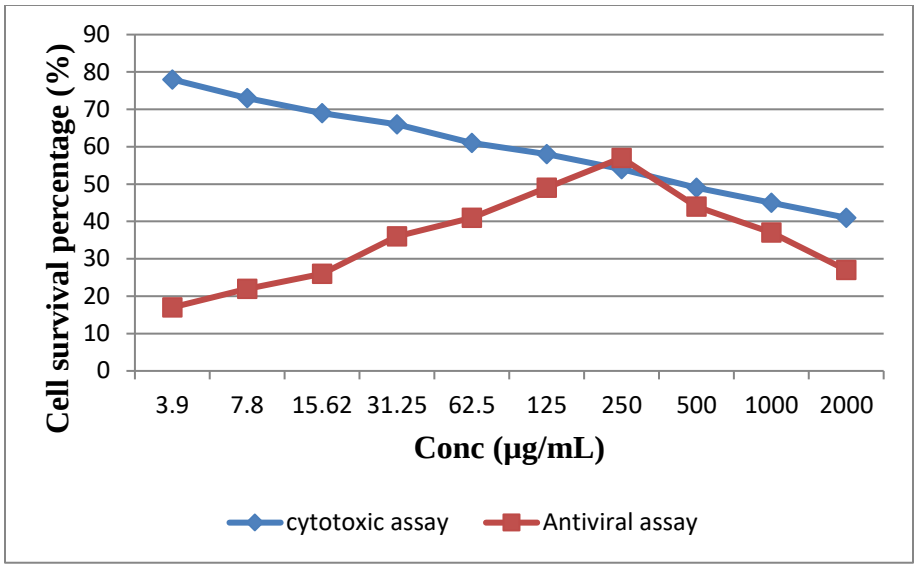

Fig 4: Comparison between antiviral and cytotoxic activities of *Olea ferruginea* Chloroform stem extract

Table 7: for BHK-21 cells the cytotoxic activity of *Olea ferruginea* leaf chloroform extract

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.498±0.04a	79
2	7.8	0.479±0.03b	76
3	15.62	0.457±0.04c	72
4	31.25	0.448±0.01c	70
5	62.5	0.421±0.02d	65
6	125	0.402±0.03e	61
7	250	0.378±0.01f	56
8	500	0.354±0.04g	52
9	1000	0.322±0.02h	46
10	2000	0.303±0.01i	42

Table 8: against FMDV the antiviral activity of *Olea ferruginea* leaf chloroform extract

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.259±0.02h	34
2	7.8	0.298±0.03f	41
3	15.62	0.339±0.02cd	49
4	31.25	0.342±0.01bc	50
5	62.5	0.356±0.01b	52
6	125	0.369±0.04a	55
7	250	0.331±0.03d	47
8	500	0.314±0.05e	44
9	1000	0.286±0.03g	39
10	2000	0.214±0.02i	25

* At 0.05 significant level the significant difference was indicated by different alphabets in the columns
* S.D means that Standard Deviation

***3.5 Olea ferruginea* alcohol stem extract:** For BHK-21 Cells, the cytotoxicity potential of alcohol *Olea ferruginea* stem extract was checked which gave CSP with respective concentration range such as 73% at 3.9µg, 72% at 7.8µg, 68% at 15.62µg/mL, 62% at 31.25µg/mL, 57% at 62.5µg/mL, 50% at 125µg/mL, 43% at 250µg/mL, 37% at 500µg/mL, 30% at 1000µg/mL and 19% at 2000µg/mL (Table 4.51, Fig 4.40). When CSP of extract was more than fifty percent (50%), it means extract was non-cytotoxic at 3.9µg/mL-125µg/ mL range of concentration. On the contrary, range of concentration from 250µg/mL to 2000µg/mL have less than fifty

percent (50%) CSP and showed that extract was cytotoxic. In addition, antiviral potential of alcohol stem extract was also observed which experienced at 50% CSP with 31.25µg/mL range of concentration (Table 4.52). While concentration ranges 2000µg/mL, 1000µg/mL, 500µg/mL, 250µg/mL, 15.62µg/mL, 7.8µg/mL, 3.9µg/mL were non-virucidal. It was found that in antiviral assay increased CSP exhibited by increased concentration range (31.25µg/mL to 250µg/mL) while decreased CSP exhibited by decreased range of concentration that were 2500µg/mL-2000µg/mL.

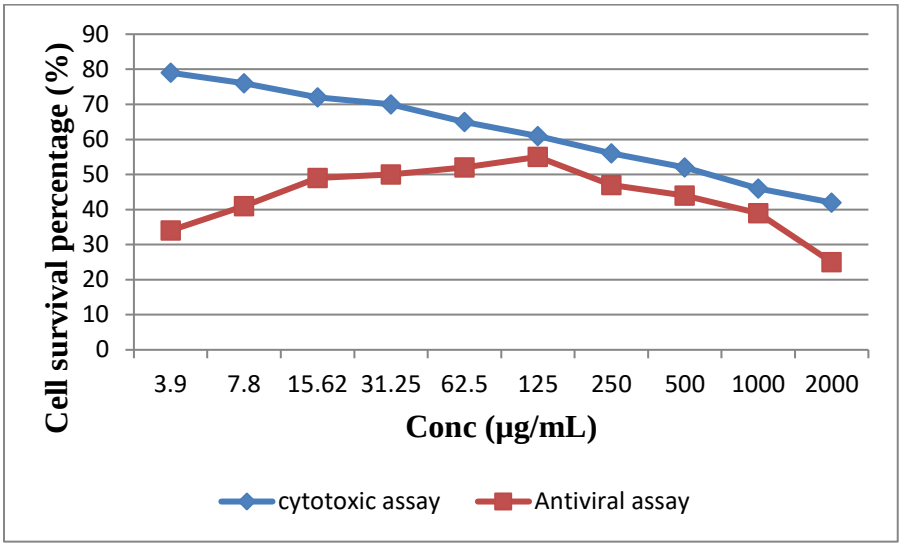

Figure 5: Comparison between antiviral and cytotoxic activity of *Olea ferruginea* chloroform leaf extract

Table 9: Cytotoxic activity of *Olea ferruginea* stem alcohol extract for BHK-21 cells

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.465±0.03a	73
2	7.8	0.458±0.02a	72
3	15.62	0.438±0.04b	68
4	31.25	0.405±0.01c	62
5	62.5	0.381±0.03d	57
6	125	0.345±0.05e	50
7	250	0.308±0.03f	43
8	500	0.274±0.03g	37
9	1000	0.238±0.02h	30
10	2000	0.184±0.04i	19

Table 10: against FMDV the antiviral activity of *Olea ferruginea* alcohol stem extract

Sr. No.	Conc. used (µg/mL)	Mean O.D. value	Cell survival percentage (%)
1	3.9	0.212±0.01g	25
2	7.8	0.298±0.03d	41
3	15.62	0.323±0.04c	46
4	31.25	0.349±0.02b	51
5	62.5	0.376±0.01a	56
6	125	0.345±0.05b	50
7	250	0.253±0.03e	33
8	500	0.229±0.05f	28
9	1000	0.204±0.02g	23
10	2000	0.178±0.02h	18

* At 0.05 significant level the significant difference was indicated by different alphabets in the columns
* S.D means that Standard Deviation

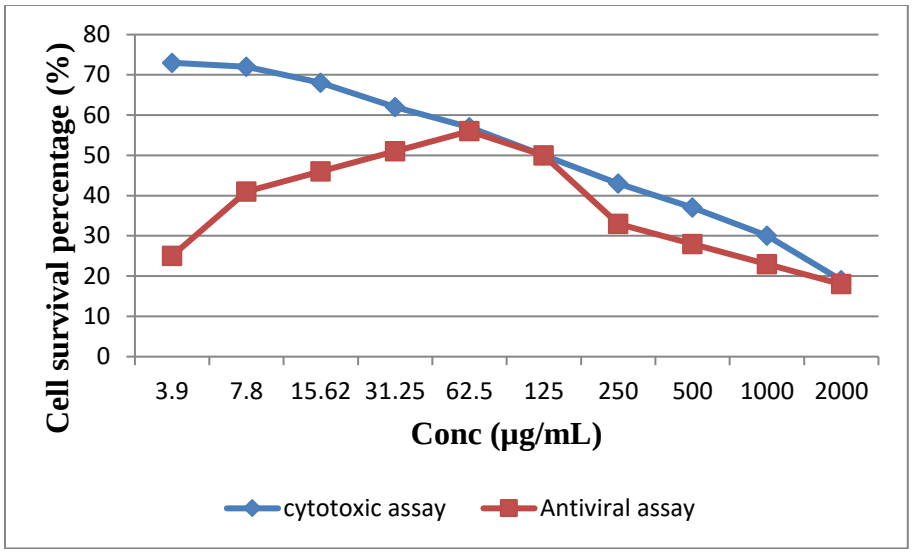

Fig 6: Comparison between the antiviral and cytotoxic activity of *Olea ferruginea* alcohol stem extract

***3.6 Olea ferruginea* leaf alcohol extract:** After stem extract cytotoxic activity, alcohol leaf extract of *Olea ferruginea* was also observed, in which CSP of extract was observed with their respective concentration ranges that are 3.9µg at76%, 7.8µg at 72%, 15.62µg/mL at 65%, 31.25µg/mL at 61%, 62.5µg/mL at 58%, 125µg/mL at 54%, 250µg/mL at 53%, 500µg/mL at 51%, 1000µg/mL at 33% and 2000µg/mL at 25% (Table 11, Fig 7). 1000µg/mL and 2000µg/mL ranges of concentration have less than fifty percent (50%) CSP that are 33% and other was 25%, except these concentration all other concentration were documented as non-cytotoxic concentration. Ranges and at these ranges the extract was found cytotoxic for BHK-21 cells. Against FMDV, 15.62µg/mL, 31.25µg/mL, 62.5µg/mL, 123µg/mL and 250µg/mL concentration were observed to know the virucidal potential of *Olea ferruginea* alcohol leaf extract and it was showed that the antiviral activity exhibited at 15.62µg/mL-125µg/mL. While maximum antiviral potential was observed at 62.5µg/mL concentration with 59% CSP (Table 12). Furthermore, results also showed that increase concentration from 500µg/mL-2000µg/mL show decreased CSP while 3.9µg/mL-7.8µg/mL concentration were documented as non-virucidal as well as non-cytotoxic.

Table 11: For BHK-21 cells the cytotoxic activity of *Olea ferruginea* leaf alcohol extract

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.482±0.01a	76
2	7.8	0.458±0.02b	72
3	15.62	0.425±0.05c	65
4	31.25	0.4±0.03d	61
5	62.5	0.388±0.02e	58
6	125	0.365±0.04f	54
7	250	0.359±0.04f	53
8	500	0.35±0.02g	51
9	1000	0.253±0.04h	33
10	2000	0.213±0.02i	25

Table 12: Antiviral activity of *Olea ferruginea* leaf alcohol extract against FMDV

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.278±0.03f	37
2	7.8	0.314±0.02e	44
3	15.62	0.354±0.05c	52
4	31.25	0.376±0.03ab	56
5	62.5	0.389±0.03a	59

6	125	0.367±0.02bc	54
7	250	0.359±0.01bc	53
8	500	0.333±0.05d	48
9	1000	0.317±0.03d	45
10	2000	0.267±0.01f	35

* At 0.05 significant level the significant difference was indicated by different alphabets in the columns
* S.D means that Standard Deviation

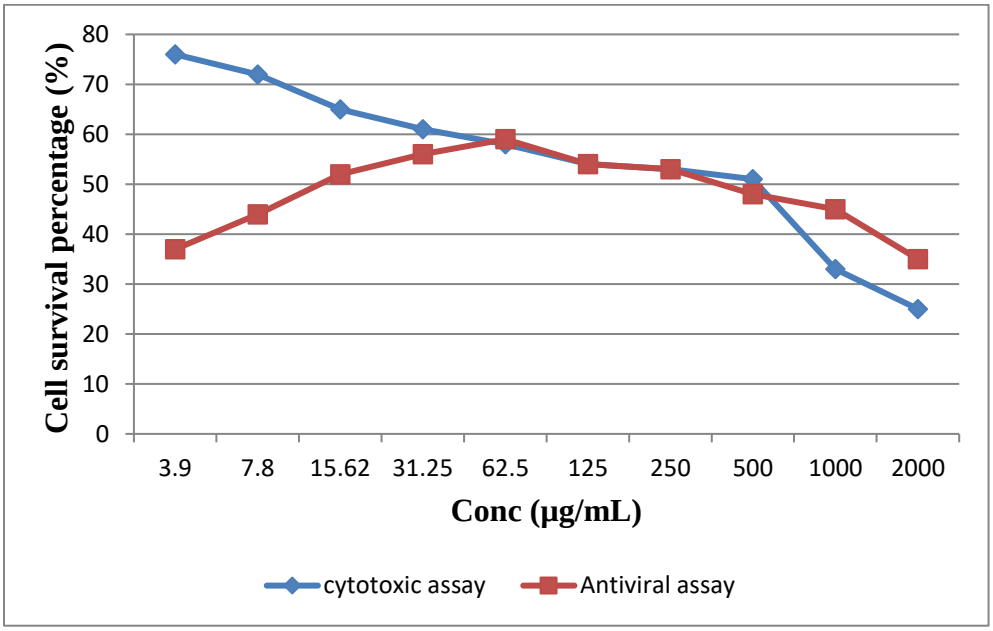

Figure 7: Comparison between the cytotoxic and antiviral activity of leaf alcohol extract of *Olea ferruginea*

***3.7 Olea ferruginea* stem aqueous extract:** Likewise, the concentration range with respective CSP was documented to analyze the cytotoxic activity of aqueous stem extract of *Olea ferruginea* for BHK-21 cells. The observed concentration and their respective CSP were 3.9µg at 75%, 7.8µg at 72%, 15.62µg/mL at 65%, 31.25µg/mL at 62%, 62.5µg/mL at 55%, 125µg/mL at 46%, 250µg/mL at 40%, 500µg/mL at 28%, 1000µg/mL at 23%and 2000µg/mL at 16% (Table 13, Fig 8). The concentration which shas more than 50% CSP was documented as a non-cytotoxic extract for BHK-21 cells and that was 3.9µg/mL to 62.5µg/mL. Whereas, the concentration such as 125µg/mL-2000µg/mL has less than 50% CSP. So, they observed as the extract had cytotoxic assay. At the concentration of 62.5µg/mL the aqueous stem extract showed antiviral activity of *Olea ferruginea* against FMDV (Table 14), while except these concentrations all other were non-virucidal.

Table 13: Cytotoxic activity of *Olea ferruginea* stem aqueous extract for BHK-21 cells

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.475±0.04a	75
2	7.8	0.459±0.02b	72
3	15.62	0.422±0.01c	65
4	31.25	0.405±0.02d	62
5	62.5	0.369±0.02e	55
6	125	0.325±0.03f	46
7	250	0.292±0.04g	40
8	500	0.227±0.02h	28
9	1000	0.204±0.02i	23
10	2000	0.166±0.04j	16

Table 14: Antiviral activity of *Olea ferruginea* stem aqueous extract against FMDV

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.145±0.03j	12
2	7.8	0.185±0.02i	20
3	15.62	0.237±0.03f	29
4	31.25	0.289±0.01d	39
5	62.5	0.345±0.04a	50
6	125	0.326±0.05b	46
7	250	0.302±0.02c	42
8	500	0.267±0.03e	35
9	1000	0.217±0.02g	26
10	2000	0.201±0.01h	23

* At 0.05 significant level, the significant difference was indicated by different alphabets in the columns
* S.D means that Standard Deviation

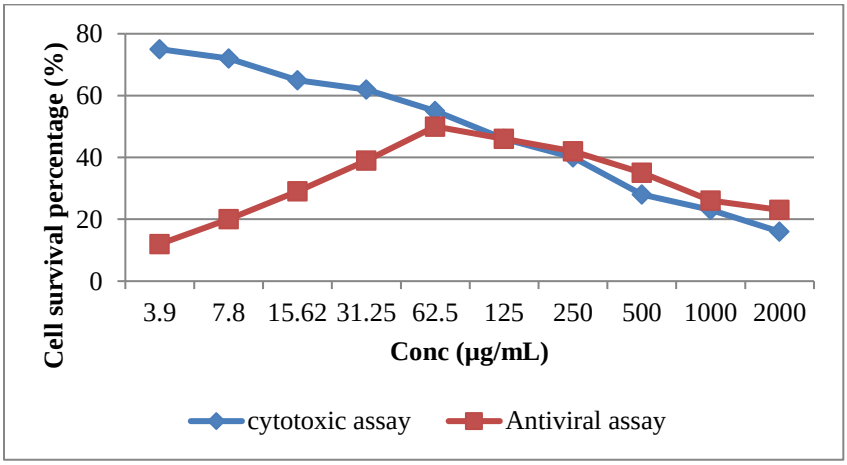

Figure 8: Comparison between the cytotoxic and antiviral activity of stem aqueous extract of Olea ferruginea

3.8 Olea ferruginea leaf aqueous extract: In the end, cytotoxic analysis was performed to observe the CSP rhrough aqueous leaf extract of *Olea ferruginea* and documented 81%-50% CSP at 3.9µg/mL concentration and showed that the 500µg/mL extract was effective for BHK-21 cells (Table 15, Fig 9). 5000µg/mL, as well as 1000µg/mL concentration, were highest ranges because their CSP was below than fifty percent (50%) and showed that the extract was cytotoxic. Meanwhile, aqueous leaf extract of *Olea ferruginea* showed an increase proportional in the effect of virucidal against FMDV with extracts increase concentration that was 62.5µg/mL at 52% CSP and 125µg/mL at 58%CSP. On the other hand, 250µg/mL or 2000µg/mL concentration ranges have less than fifty percent (50%) CSP (Table 16). Lower ranges of concentration showed that the extract was non-cytotoxic but non-vircucidal i.e. 3.9µg/mL to 31.25µg/mL.

Table 15: for BHK-21 cells the cytotoxic activity of *Olea ferruginea* leaf aqueous extract

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.505±0.01a	81
2	7.8	0.495±0.05b	79
3	15.62	0.461±0.02c	72
4	31.25	0.435±0.01d	67
5	62.5	0.403±0.02e	61
6	125	0.386±0.04f	58
7	250	0.367±0.05g	54
8	500	0.345±0.02h	50
9	1000	0.278±0.03i	37
10	2000	0.211±0.02j	24

Table 16: Against FMDV the antiviral activity of *Olea ferruginea* aqueous leaf extract

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.167±0.02a	16
2	7.8	0.232±0.03i	28
3	15.62	0.274±0.04g	37
4	31.25	0.314±0.01e	44
5	62.5	0.354±0.02c	52
6	125	0.384±0.03b	58
7	250	0.312±0.03c	44
8	500	0.304±0.01d	42
9	1000	0.256±0.03f	33
10	2000	0.214±0.05h	25

* At 0.05 significant level the significant difference was indicated by different alphabets in the columns
* S.D means that Standard Deviation

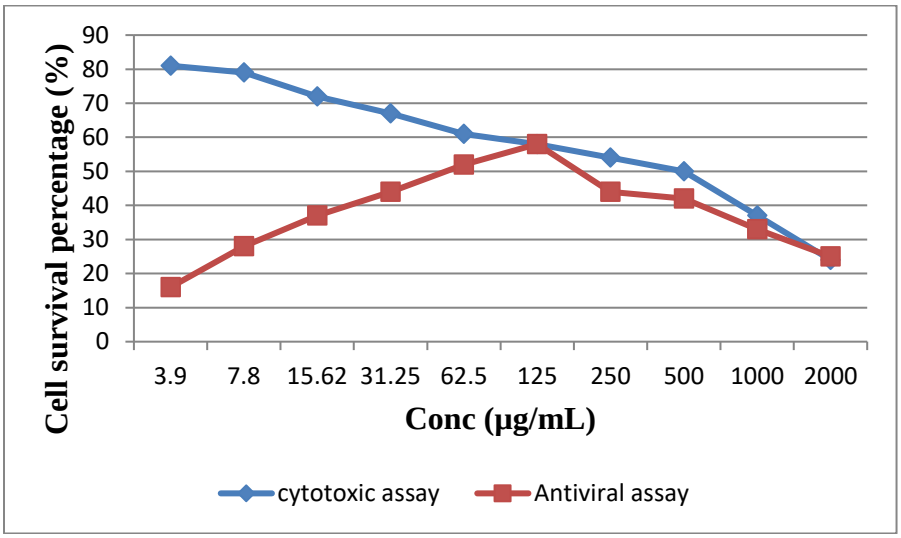

Fig 9: Comparison between the antiviral and cytotoxic activity of *Olea ferruginea* aqueous leaf extract

4 Discussion and conclusion: In the present study, stem and leaf extracts of *Olea ferruginea* Royle plant were used to check its cytotoxic effect as well as antiviral potential by BHK-21 cells. The extract of n-hexane stem showed toxicity at concentration range of 31.25 µg/mL-125 µg/mL while showed cytotoxicity at 3.9µg/mL to 125µg/mL along with extract showed CSP 54% at 62.5µg/mL or 58% CSP 125atµg/mL concentration respectively (Table 1, Table 2). Similarly, cytotoxicity potential of chloroform extract was measured at 500 to 2000 µg/mL along with extract was virucidal at 125µg/mL concentration against FMDV, while at 31.25 µg/mL-250 µg/mL concentration range the chloroform leaf extract showed its antiviral potential (Fig 3). Likewise, antiviral significant potential was documented by *Olea ferruginea* Royle's alcohol leaf extract at 15.62µg/mL-250µg/mL concentration with 52% to 59% CSP while cytotoxic activity of leaf extract was observed at 1000 µg/mL to 2000µg/mL. In the same way, water leaf extract of *Olea ferruginea* Royle was observed as virucidal at 62.5µg/mL concentration. Cytotoxic activity of leaf extract was higher at higher concentration that was 1000µg/mL and 2000µg/mL. Younus *et al.*, (2017) reported in their study that the cytotoxicity activity of *Moringa Oleafera*'s ethanolic extract at more than 100 µg/mL concentration for CORL-23 or PC3 cancer cells as well as for 10FS normal cells. So, their findings are similar to an investigation of the present study. In the same way, Gopalakrishnan *et al.*, (2016) studied the cytotoxicity of the aqueous *Moringa Oleafera*'s leaf extracts on the Hella cells which caused a decrease percentage in cell survival at a concentration of 100 µg/mL, phenolic compounds became a cause of cytotoxic potential at higher concentration related to various *Olea ferruginea* Royle's extract (Leone *et al*. 2015) and it was also observed that extracts from stems possessed less antiviral potential as compared to leaf extract. Maybe the occurrence of few kinds of phytochemicals for example polyphenol became a reason for cytotoxicity at higher concentrations for stem extract. In the same way, various studies also reported that the cytotoxic effect of various other polyphenols might be the reason at a higher

concentration for their action of prooxidant (Hossen *et al*. 2020; Makarewicz, 2021)

In short, Imadi *et al*. (2018) explained that plants were used as a source of medicine by people of rural or tribal areas with the help of experienced and generation of knowledge. So, the findings of this study were better than Bastos *et al*. (2013) study who did not report any antiviral potential of 21 different plants' extract against FMDV. Similarly, in this study, the rise concentration showed the increase of cytotoxic activity of the extract. Nasirzadeh and Jafer (2018) reported that leaf of *Olea ferruginea* Royle have antiviral potential which was due to the main constituents of plant extract named Oleuropein, and the antiviral potential of Oleuropein was documented against the virus of hepatitis B (Rasheed *et al.*, 2020), also for the virus of human immunodeficiency (Takeda, *et al*. 2021) as well as against hemorrhagic septicemia rhabdovirus reported by Barbaro *et al*. (2014).

It is concluded from the discussion that the antiviral medicine can be prepared according to the concentrations at which the extract is antiviral and non-cytotoxic. As we increase the concentration of the extract, it shows good antiviral results but with the increase in the concentration the survival percentage of the cells was decreased. So, the range of the extract which is suiTable for the survival of cells and shows good antiviral activity can be used by the pharmaceutical industries

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