ANTVIRAL ACTIVITY OF *OLEA EUROPAEA* L. AGAINST FOOT AND MOUTH DISEASE VIRUS

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ABSTRACT: This research had been conducted to analyze the natural agents for the antiviral activity that utilized essentially and simply in the field or it had the objective to lessen the animals' carrier condition as well as to decrease infection during an outbreak of disease or infected animals' quantity. The antiviral and cytotoxic effects of *Olea europaea* L. against foot and mouth disease virus (FMDV) were observed by using the cell culture technique. FMD virus is considered as the causal agent of the disease which influences domesticated livestock and became a cause of intense sickness that is characterized by low morality, high dismalness of teats, tongue, and nose, fever, vesicular sores on feet, and weakness. It was predicted that the *Olea europaea* L.'s alcohol leaf extracts had antiviral potent activity at 31.25Hg/mL-250Hg/mL concentration range along with a range of CSP that was fifty-one percent (51%) to sixty-three percent (63%) which followed by chloroforms extract activity in which percentage of cell survival was analyzed fifty-four percent (54%) or fifty-seven (57%) at concentration 31.25 Hg/mL or 62.5 Hg/mL respectively. So, the antiviral activity of *Olea europaea* L. observed in this research might be due to the presence of oleuropein which inhibited the *in vitro* infectivity of the Foot and mouth disease virus (FMDV).

Keywords; Antiviral, *Olea europaea*, FMDV, Vitro.

(Received 23.04.2021 Accepted 05.06.2021)

INTRODUCTION

Olea europaea L. with a rounded crown commonly called olive that grows in the Mediterranean region. Flowers of *Olea europaea* L. are scented and show drupes of oval green that are olives more than 1.5 inches long and ripen to black. Moreover, authors stated that the *Olea europaea* L. products are being used as purgative, tonic, sedative, soothing, aphrodisiac, and nutritive drugs (Dekdouk *et al*., 2015). Traditionally, the oil of *O. europaea* is used for treatment in a specific condition such as hypertension, colic sciatica, and paralysis (Ben Salem *et al*., 2015; Pošćić *et al.,* 2018; Ab Muib *et al.,* 2020).

Wild animals, cattle, pigs, goat, and sheep have various infectious diseases in which foot and mouth diseases i.e. FMD is common and caused by a kind of virus. FMD virus is considered as the causal agent of this disease which influences the domesticated livestock and became a cause of intense sickness that is characterized through low morality, high dismalness of teats, tongue, and nose, fever, vesicular sores on feet, and weakness. Moreover, genetically the causative agent of the disease is varied and having seven serotypes, in which every serotype has vaccination from the prevention of infection and that vaccination is different or not recoup against other Serotype's contamination (Poonsuk, Giménez-Lirola and Zimmerman, 2018). In previous studies it is stated that the best vaccine of FMDV based on chemically inactivated foot and mouth diseases virus (FMDV), assured complete protection just after seven days after vaccination (Deshpande and Chaphalkar, 2013; Varshovi *et al.,* 2017). This is because the expected time to trigger an insusceptible reactions, as well as generally these this vaccination, gave protection against one or also few of distinct sixty distinctive FMDV serotypes (Deshpande and Chaphalkar, 2013; Norian and Azadmehr, 2017). Moreover, animals called the carrier and also create a state that is complicated during outbreak situations because they have chances to get infected again even after the vaccination (El-Khabaz and Al-Hosary, 2017). An assay of cytotoxicity was cost effective and rapid method to analyze the failures that possibly occurred before the compound was being checked for its activity through a costly process of development (Tomar, 2018). The problem which is going to be addressed in this research is that the animals faced FMDV disease which badly affect the domesticated animals and have no proper vaccination (Gong *et al.,* 2019). It will be recovered with help of plant that considered as medicated plants. It 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 (Imadi *et al.,* 2018). 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). *Olea europaea* L. plant in this study used to control the disease because it is cost effective method. This research has objectives to analyze the natural agents for antiviral that utilized essentially and simply in the field or have objectives to lessen the animals' carrier condition as well as to decrease their situation during the outbreak of disease and the number of infected animals. Furthermore, this research highlights the antiviral and cytotoxic affects of the plant *O. europaea* against the virus of FMD with the help of cell culture techniques. This study is also significant for economic point of view because FMDV affect the domesticated the animals which adverse the economic sector, so; urgent and cost effective control is necessary. The study was done on the Alhagi maurorum Medik's extracts which used in Iran to treat the FMDV with its antiviral capability that showed that it treat the FMDV (Younus *et al.,* 2017). But in present study *Olea europaea* L. extract used to show its antiviral and cytotoxic activity against FMDV. This medicated plant was not previously studied in any research for the FMDV. Non-polar and polar solvents have four distinct extracts of leaf and stem of plants that were used. Furthermore, it was also recommended that the combination of antiviral agents and vaccine which considered as a more effective strategy to treat animals that are contaminated by FMDV; however, currently there are no endorses against drugs of FMD for the treatment purpose of FMDV. Likewise, researchers also studied the relation of antiviral effects of synthetic compounds with natural compounds (Wachsman *et al.,* 1998; Deshpande and Chaphalkar, 2013) . Antiviral agents the disrupt the process of virus replication in infected cells along with it also inhabit the adhesion and synthesis of mRNA or gave relief from the coat of protection (YILMAZ, Taşbozan and Erbaş, 2018).

Fig 1. Plate: *Olea europaea* L.

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:

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

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 impacts (CPE) eighty to nighty percent (80-90%) of the Inoculated cells were observed under the microscope (inverted) regularly while CPE was observed on the 6^{th} 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 suspension of viral was transmitted to the eppendorf **tu**be, after that the suspension at 5000 rpm was centrifuged at 4ºC for 5 to10 min. this method separates at the bottom, a cell's pellet of debris. After that, the supernatant was stored until it was used at a temperature of -70ºC.

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 the virus, while the last two wells that contained maintenance media and cells of each plate were used as control. Then, under the inverted type of 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. Walls that were infected by the virus were compared with the wall that controls the cells and then marked either they were positive or negative (Reed and Muench, 1938).

Following formula 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. MTT assay 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 of human carcinoma cells was checked by using MTT assay of standard colorimetric (Akhir *et al.*, 2011).

***3.1 n*-hexane extract *of Olea europaea* L. stem:** First of all, for BHK-21 cells the cytotoxicity of extract *Olea europaea* L. n-hexane stem was observed at a different concentration that showed in Table 1. At 500Hg/mL – 2000 Hg/mL, the extract showed cytotoxic, while CSP showed the range less than fifty percent (50%). So, the 3.9 Hg/mL --- 250 Hg/mL concentration range was considered as non-cytotoxic. It is because this CSP has more than fifty percent (50%) at that range. While the *O. europea* stem's n-hexane extract was revealed at 31.25Hg/mL or 62.5Hg/mL. The antiviral activity with fifty-two percent (52%) and fifty-six percent (56%) CSP respectively showed in Table 2.

While the comparison of Cytotoxicity and antiviral is depicted in Figure 1. Moreover, at low 152.67Hg/mL, 7.8Hg/mL, and 3.9 Hg/mL concentration range the extract was showed their non- virucidal activity against FMDV along with below fifty percent (50%) CSP. Similarly, 125 Hg/mL - 2000 Hg/mL are higher concentration ranges and considered as non-virucidal as well as cytotoxic (Fig 1).

***3.2* O. europaea leaf *n*-hexane extract:** Secondly, experiment was done to analyze the cytotoxic for cell survival of n-hexane extract for *O. europaea* for BHK-21 cell and obtained 84%, 83%, 82%, 79%, 73%, 67%, 51%, 39%, 27% and 10% at 2000µg/mL, 1000µg/mL, 500µg/mL, 250µg/mL, 125µg/mL, 62.5µg/mL, 31.25µg/mL, 15.62µg/mL, 7.8µg and 3.9µg respectively (Table 3, Fig 2). Along with CSP that was more than fifty percent (50%), the non-cytotoxic extract has occurred at 3.9 Hg/mL-250Hg/mL concentration range. While cytotoxicity at 500Hg/mL to 2000Hg/mL was obtained with less than 50% CSP. In the same way, the antiviral activity of O. europaea leaf's n-hexane extract was also observed with CSP at the same concentration, and that was 52%, 57%, 63%, 67%, 69%, 66%, 62%, 48%, 40% and 33% for analysis of cytotoxicity, (Table 4, Fig 2). Concentration range was found as virucidal i.e. 3.9Hg/mL-250Hg/mL against virus disease of foot and mouth, it is because at this range the CSP observed more than 50%. On the other hand, at 500Hg/mL-2000Hg/mL the effect of non-viral was observed along with CSP forty-eight percent (48%) and thirty-three percent (33%) respectively.

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

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.528±0.04a	85
2	7.8	0.505±0.03b	81
3	15.62	0.485±0.03c	77
4	31.25	0.445±0.04d	69
5	62.5	0.391±0.02e	59
6	125	0.372±0.01f	55
7	250	0.355±0.03f	52
8	500	0.328±0.04g	47
9	1000	0.317±0.03g	45
10	2000	0.299±0.05h	41

Table 2: Against FMDV the Antiviral activity of *n*-hexane extract *O. europaea* stem.

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.528±0.03e	37
2	7.8	0.505±0.06cd	41
3	15.62	0.485±0.04c	44
4	31.25	0.445±0.05b	54
5	62.5	0.391±0.03a	57
6	125	0.372±0.02c	44

7	250	0.355±0.04d	40
8	500	0.328±0.04f	33
9	1000	0.317±0.02g	26
10	2000	0.299±0.02h	23

* in the columns different alphabets indicate at 0.05 significant level the significant difference

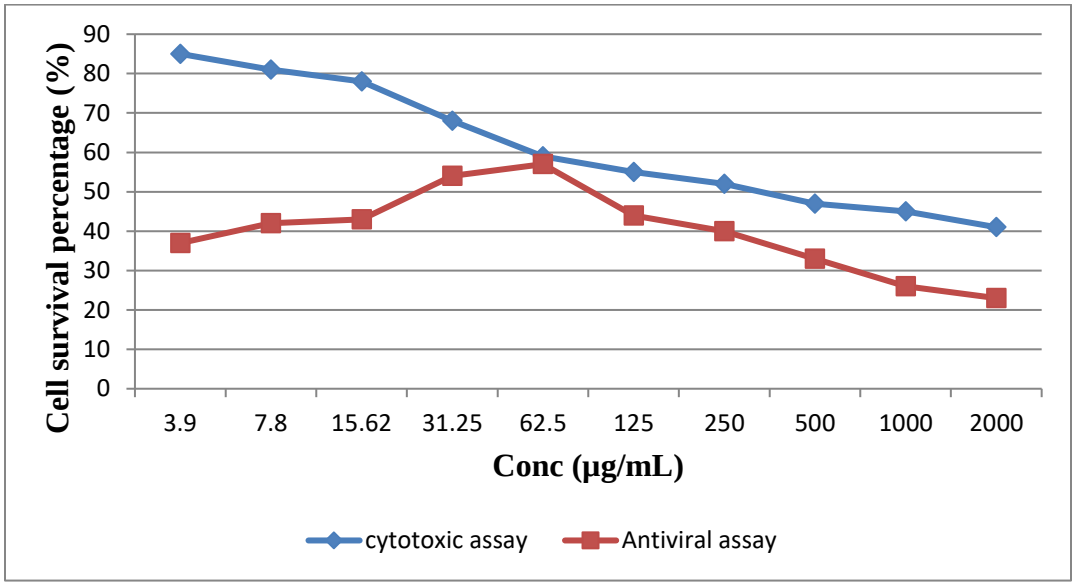

Fig 1: Comparison between the antiviral and cytotoxic activity of *O. europaea n*-hexane stem extract

Table 3: for BHK-21 cells *the* cytotoxic activity of *O. europaea* leaf *n*-hexane extract.

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.52±0.05a	84
2	7.8	0.517±0.01ab	83
3	15.62	0.511±0.02b	82
4	31.25	0.498±0.04c	79
5	62.5	0.467±0.022d	73
6	125	0.435±0.01e	67
7	250	0.348±0.01f	51
8	500	0.285±0.05g	39
9	1000	0.226±0.03h	27
10	2000	0.137±0.02i	10

Table 4: Against FMDV *the antiviral activity of O. europaea* leaf *n*-hexane extracts

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.243±0.02g	31
2	7.8	0.297±0.01e	41
3	15.62	0.339±0.03c	49
4	31.25	0.345±0.03bc	50
5	62.5	0.354±0.02ab	52
6	125	0.363±0.01a	54
7	250	0.334±0.04c	48
8	500	0.312±0.02d	44
9	1000	0.274±0.03f	37
10	2000	0.244±0.01g	31

* in the columns different alphabets at 0.05 significant level indicate the significant difference

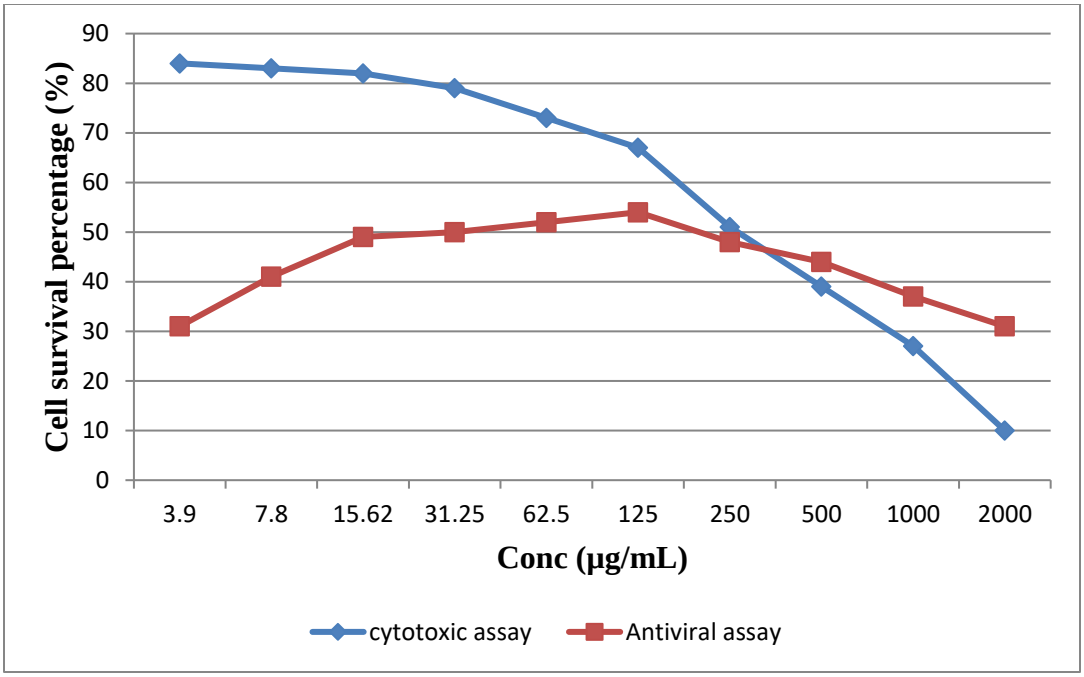

Fig 2: Comparison between the antiviral and cytotoxic activity of *O. europaea n*-hexane leaf extract

***3.3* chloroform extract *of O. europaea* stem:** After that, BHK-21 cells' cytotoxic analysis of chloroform *O. europaea* stem extract was measured at different concentrations that were 2000µg/mL, 1000µg/mL, 500µg/mL, 250µg/mL, 125µg/mL, 62.5µg/mL, 31.25µg/mL, 15.62µg/mL, 7.8µg and 3.9µg, while its CSP was 48%, 51%, 53% 56%, 58%, 61%, 64%, 67%, 70%, and 73% respectively (Table 5, Fig 3). It was concluded that at all concentrations the extract showed non-cytotoxic activity except at 2000Hg/mL. survival of cell in this antiviral assay for extract related to stem hexane *O. europaea* against foot and mouth disease (Table 6). Likewise, 62.5Hg/mL-250Hg/mL concentration range was found as antiviral with CSP at this range was more than fifty percent (50%). On the other hand, high concentration was analyzed as non-cytotoxic such as 500Hg/mL-1000Hg/mL along with CSP less than 50 % was observed. So, such concentration was observed as non-effective against disease virus. 2000Hg/mL concentration was cytotoxic along with non-virucidal showed in Figure 3.

Table 5: chloroform extract of *O. europaea* stem's Cytotoxic activity for BHK-21 cells

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.467±0.02a	73
2	7.8	0.451±0.03b	70
3	15.62	0.431±0.01c	67
4	31.25	0.416±0.02d	64
5	62.5	0.401±0.03e	61
6	125	0.388±0.04f	58
7	250	0.375±0.03g	56
8	500	0.361±0.05h	53
9	1000	0.349±0.03h	51
10	2000	0.335±0.02i	48

Table 6: *Olea europaea* L. stem chloroform's Antiviral activity extract against FMDV.

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.211±0.05g	24
2	7.8	0.256±0.02f	33
3	15.62	0.287±0.01e	39

4	31.25	0.335±0.03c	48
5	62.5	0.343±0.02bc	50
6	125	0.35±0.04b	51
7	250	0.364±0.02a	54
8	500	0.314±0.03d	44
9	1000	0.267±0.04f	35
10	2000	0.216±0.02g	25

* In the columns different alphabets at 0.05 significant level indicate the significant difference

* S.D.= Standard Deviation

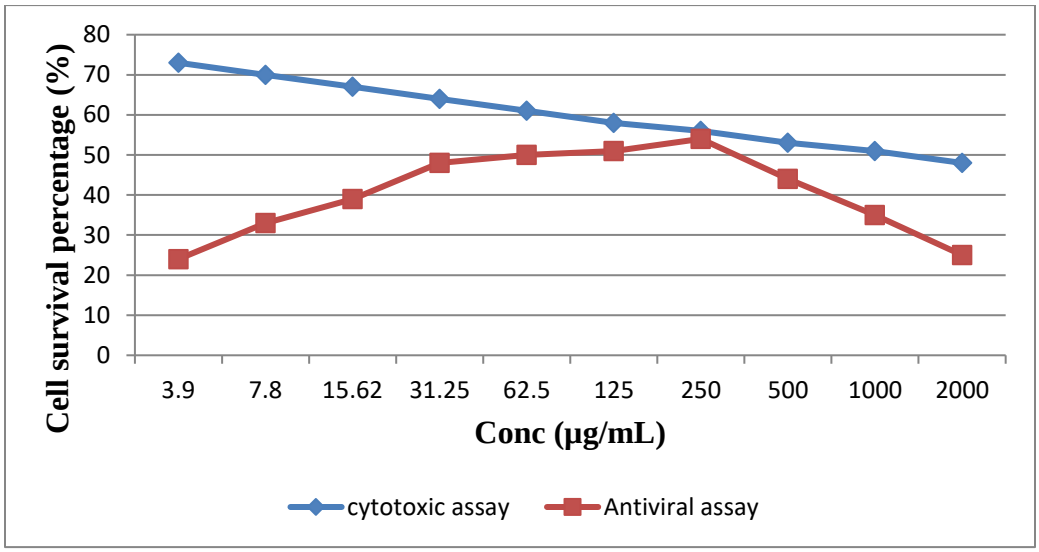

Fig 3: Comparison between antiviral and cytotoxic of *O. europaea* chloroform stem extract.

3.4 Extract of *O. europaea* leaf chloroform: After the *O. europaea* stem chloroform extract for leaf was observed. Cytotoxic assay for cell survival of leaf extract for BHK-21 cell was observed and that ranging between eight to forty-six (8% to 46%) ascending to a respective range of concentration (Table 7 Fig 4). If CSP was analyzed more than fifty percent (50%) then the range of concentration that was 3.9Hg/mL-500Hg/mL was observed safe for cell *i.e.* BHK-21, while extract considered toxic with concentration 1000Hg/mL and 2000Hg/mL for cell with CSP which recorded as less than fifty percent (50%). Chloroform leaf extract was checked for this activity as antiviral against FMDV at concentrations of 2000µg/mL, 1000µg/mL, 500µg/mL, 250µg/mL, 125µg/mL, 62.5µg/mL, 31.25µg/mL, 15.62µg/mL, 7.8µg and 3.9µg, the range that observed virucidal was 31.25µg/mL to 250µg/mL as CSP was above 50% (Table 4).

Table 7: For BHK-21 cells the cytotoxic activity of *O. europaea* chloroform leaf extract.

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.499±0.01a	80
2	7.8	0.491±0.03a	78
3	15.62	0.474±0.03b	75
4	31.25	0.458±0.02c	72
5	62.5	0.431±0.02d	67
6	125	0.418±0.05e	64
7	250	0.382±0.04f	57
8	500	0.361±0.02g	53
9	1000	0.341±0.04h	49
10	2000	0.325±0.02i	46

* In the columns, different alphabets at 0.05 significant level indicate the significant difference

* S.D means Standard Deviation

Table 8: Against FMDV the antiviral activity of *O. europaea* extract leaf chloroform extract.

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.216±0.03g	25
2	7.8	0.238±0.02f	30
3	15.62	0.289±0.01e	39
4	31.25	0.354±0.01b	52
5	62.5	0.376±0.04a	56
6	125	0.388±0.03a	58
7	250	0.359±0.05b	53
8	500	0.339±0.04c	49
9	1000	0.312±0.04d	44
10	2000	0.289±0.05e	39

* In the columns, different alphabets at 0.05 significant level indicate the significant difference
* S.D means Standard Deviation

At 500µg/mL - 15.62µg/mL and 3.9µg/mL ranges, the extract of the leaf did not show any antiviral activity so it observed as non-toxic at these points while ranges that were 1000µg/mL and 2000µg/mL was found to be non-viral as well as cytotoxic (Table 8 and Fig 4).

***3.5* Alcohol extract *of O. europaea* stem:** Respectively, the cell survival have percentage in extract of *O. europaea* stem alcohol and the assay of cytotoxic was observed at 22%, 29%, 36%, 40%, 44%, 58%, 63%, 67%, 70% and 71% at concentration of 2000µg/mL, 1000µg/mL, 500µg/mL, 250µg/mL, 125µg/mL, 62.5µg/mL, 31.25µg/mL, 15.62µg/mL, 7.8µg and 3.9µg (Table 9). The safe range of concentration against BHK-21 was 3.9µg/mL - 62.5µg/mL or at this range the CSP was greater than fifty percent. As the concentration in cytotoxic assay increased, the CSP decreased. Against FMDV, the antiviral activity of *O. europaea* alcohol stem extract was checked which found to be 10%, 16%, 19%, 43v, 49%, 54%, 50%. 46%, 27% and 22%22%, 27%, 46%, 50%, 54%, 49%, 43%, 19%, 16% and 10% at concentration 2000µg/mL, 1000µg/mL, 500µg/mL, 250µg/mL, 123µg/mL, 62.5µg/mL, 31.25µg/mL, 15.62µg/mL, 7.8µg and 3.9µg respectively (Table 10, Fig 5). With CSP above fifty percent the extract was non cytotoxic and antiviral at the concentration of 31.25µg/mL to 62.5µg/mL. Alcohol stem extract of *O. europaea* did not showed, at 125µg/mL to 2000µg/mL concentration ranges, any antiviral activity as shown in results of Table 10. The concentration of CSP was less than fifty percent (50%); so, the extract against FMDV was considered ineffective at above mention concentrations.

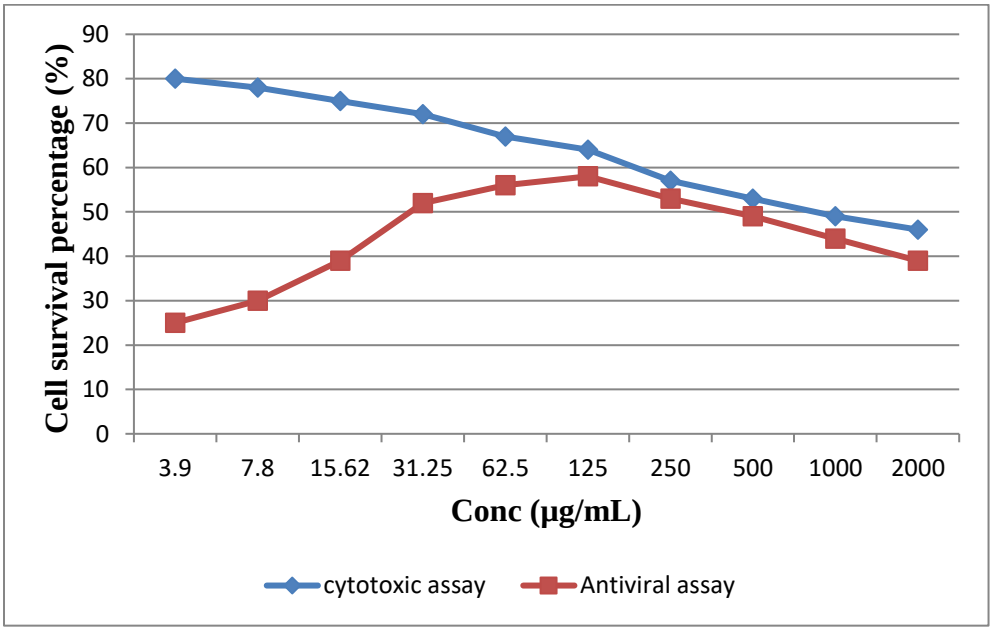

Fig. 4: Comparison between the antiviral and cytotoxic activity of leaf chloroform extract of *O. europaea*

Table 9: For BHK-21 cells the cytotoxic activity of *O. europaea* alcohol stem extract.

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.455±0.02a	71
2	7.8	0.449±0.02a	70
3	15.62	0.432±0.04b	67
4	31.25	0.412±0.03c	63
5	62.5	0.385±0.04d	58
6	125	0.315±0.01e	44
7	250	0.293±0.06f	40
8	500	0.271±0.03g	36
9	1000	0.234±0.07h	29
10	2000	0.198±0.03i	22

Table 10: Against FMDV the Antiviral activity of *O. europaea* alcohol stem extract.

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.198±0.04f	22
2	7.8	0.226±0.02e	27
3	15.62	0.321±0.01c	46
4	31.25	0.346±0.02b	50
5	62.5	0.365±0.03a	54
6	125	0.341±0.02b	49
7	250	0.309±0.02d	43
8	500	0.184±0.03g	19
9	1000	0.168±0.02h	16
10	2000	0.135±0.01i	10

* In the columns, different alphabets at 0.05 significant level indicate the significant difference
* S.D means Standard Deviation

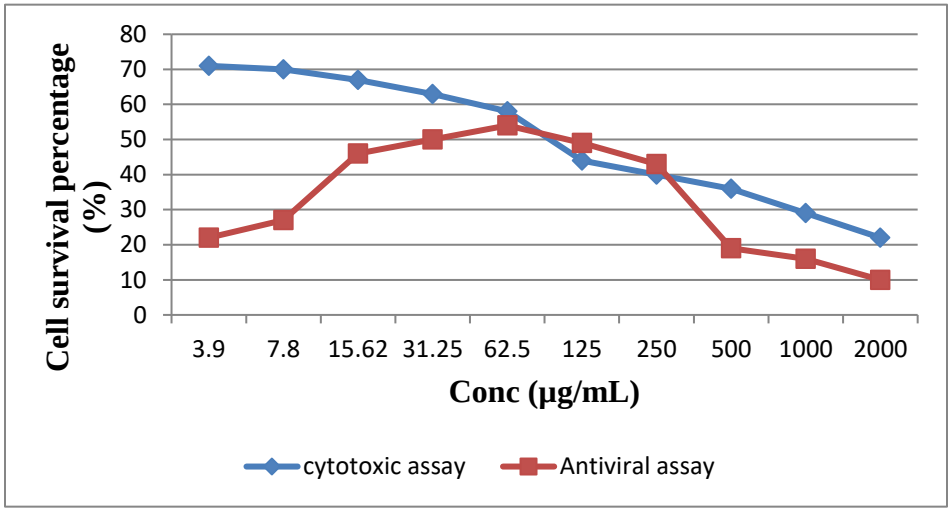

Fig 5: Comparison between the antiviral and cytotoxic activity of *O. europaea* stem alcohol extract

At low concentration, no antiviral activity was observed and that ranges were 15.62µg/mL, 7.8µg/mL, and 3.9 µg/mL. Likewise, the increased concentrations from 31.25µg/mL to 62.5µg/mL showed an increase in the range of concentration of CSP while further increase in concentration gave the result as decrease in CSP (Table 10, Fig 5).

***3.6 Alcohol leaf extract of O. europaea*:** The cell survival in cytotoxic assay of alcohol leaf extract of *Olea europaea* L. along with antiviral activity of *Olea europaea* L. against BHk-21 cells was 30%, 37%, 44%, 53%, 56%, 62%, 65%, 71%, 75%, and 81% at concentrations of 2000µg/mL, 1000µg/mL, 500µg/mL, 250µg/mL, 125µg/mL, 62.5µg/mL, 31.25µg/mL, 15.62µg/mL, 7.8µg and 3.9µg respectively (Table 11).

The safe range of concentration for BHK-21 cells is 3.9µg/mL to 250µg/mL and at these concentrations, the CSP for BHK-21 cells was more than fifty percent (50%). CSP decreased with an increase in the concentration of cytotoxic assay (Fig 6). When alcohol extract against FMDV for antiviral activity of *O. europaea* was checked, the CSP at their respective concentrations was observed to be 41%, 44%, 49%, 63%, 60%, 56%, 51%, 39%, 33%, 30% (Table 12, Fig 6). The extract did not show any activity related to antiviral at concentrations that were termed as non-cytotoxic and that was 3.9µg/mL because at this rate the CSP was less than fifty percent (50%). However, the extract was observed as effective at concentration range 31.25 µg/mL - 250µg/mL against the virus; so, there was a proportional increase in CSP for antiviral assay. However; an increase in concentration at 500µg/mL to 2000µg/mL gave result of a decrease in CSP in the assay (Fig 6).

Table 11: Cytotoxic activity of *O. europaea* alcohol leaf extract for BHK-21 cells.

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.49±0.01a	81
2	7.8	0.473±0.,03b	75
3	15.62	0.452±0.02c	71
4	31.25	0.421±0.05d	65
5	62.5	0.405±0.05e	62
6	125	0.375±0.04f	56
7	250	0.359±0.05g	53
8	500	0.315±0.02j	44
9	1000	0.276±0.04i	37
10	2000	0.238±0.03j	30

Table 12: Against FMDV the antiviral activity of *O. europaea* alcohol leaf extract.

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.236±0.02i	30
2	7.8	0.254±0.02h	33
3	15.62	0.287±0.05g	39
4	31.25	0.351±0.04d	51
5	62.5	0.378±0.,03c	56
6	125	0.399±0.06b	60
7	250	0.415±0.02a	63
8	500	0.339±0.01d	49
9	1000	0.312±0.01e	44
10	2000	0.298±0.04f	41

* In the columns different alphabets at 0.05 significant level indicate the significant difference

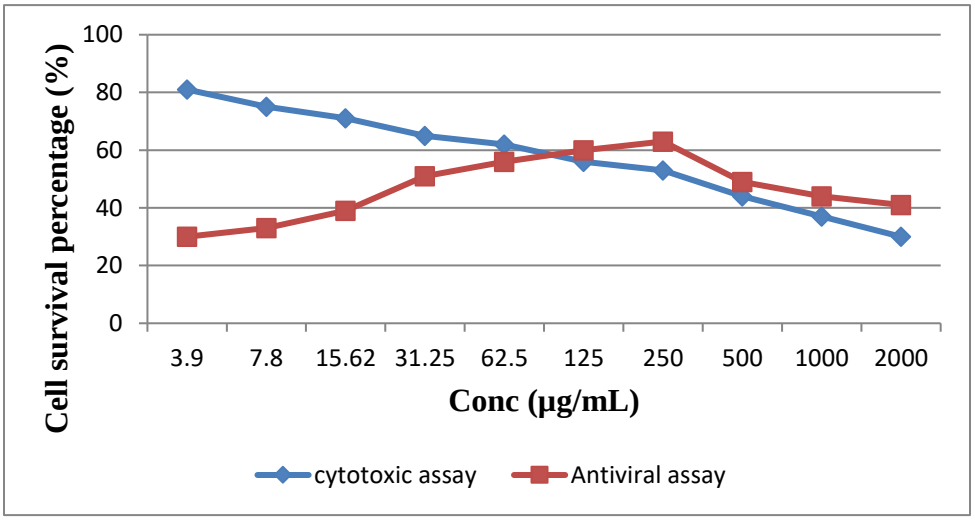

Fig 6: Comparison between the cytotoxic and antiviral activity of leaf alcohol extract of *O. europaea*

3.7* Aqueous *stem extract of O. europaea: In cytotoxic analysis, the CSP for aqueous stem extract of *O. europaea* was measured as 24%, 28%, 31%, 35%, 51%, 52%, 54%, 58%, 61% and 67% at concentrations of 2000µg/mL, 1000µg/mL, 500µg/mL, 250µg/mL, 125µg/mL, 62.5µg/mL, 31.25µg/mL, 15.62µg/mL, 7.8µg and 3.9µg respectively (Table 13). Likewise, analysis of cytotoxic was revealed that 3.9µg/mL to 62.5µg/mL ranges of the extract was noncytotoxic and all these concentrations ranges have CSP more than fifty percent (50%), thus indications showed that extract of the stem to be observed as safe for BHK-21 cells. CSP was observed less than fifty percent (50%) at 125µg/mL to 2000µg/mL concentration which representing the extract's toxicity activity for BHK-21 cells. Against FMDV the extract of aqueous *O. europaea* stem did not show for antiviral any significant potential**.** Survival of Cell has ranged in the analysis of antiviral that was 17%, 24%, 25%, 27%, 31%, 51%, 48%, 41%, 37% and 25% which were similar to the concentration that achieved during cytotoxic analysis (Table 14, Fig 7).

On the other hand, at a concentration range that was 3.9µg/mL - 31.25µg/mL the extract was observed as non-cytotoxic as well as did not showed any activity of antiviral. The concentration 62.5µg/mL was analyzed as effective against the foot and mouth disease virus (FMDV). Likewise, Figure 7 showed a decreasing the effect of virucidal at the highest extract's concentration.

***3.8 O. europaea* leaf aqueous extract:** At last, analyses were applied to measure the Cytotoxic leaf aqueous' extract of *O. europaea* for BHK-21 cells which indicated that the percentage range of cell survival's concentration-lied within 84 to 44%. Concentration range was observed as non-cytotoxic that are 500µg/mL, 250µg/mL, 125µg/mL, 62.5µg/mL, 31.25µg/mL, 15.62µg/mL, 7.8µg and 3.9µg because CSP that observed at above concentrations was above 50%. With the decreased value of CSP, the concentration of the extract was also observed in increased ranges which indicated that the higher concentration was cytotoxic and that was more than 500µg/mL to BHK-21 cells shown in Table 15, Fig 8. Against FMDV, the antiviral assay of *O. europaea* leaf aqueous extract gave the result 26%, 33%, 44%, 48%, 53%, 52%, 50%, 44%, 34% and 29%. 31.25µg/mL to 125µg/mL as cell survival ranges. The concentration range was virucidal because its CSP at these mention concentrations was more than fifty percent (50%) (Table 16). Furthermore, results showed that extract was observed as non-virucidal at concentrations range of 250µg/mL to 2000µg/mL. The highest potential for virucidal was observed at 125µg/mL concentration range with CSP was 53% (Fig 8).

Table 13: For BHK-21 cells the cytotoxic activity of *O. europaea* stem aqueous extract.

Sr. No.	Conc. used (µg/mL)	Mean O.D. value	Cell survival percentage (%)
1	3.9	0.435±0.01a	67
2	7.8	0.401±0.04b	61
3	15.62	0.385±0.04c	58
4	31.25	0.367±0.02d	54
5	62.5	0.355±0.01e	52
6	125	0.349±0.03e	51
7	250	0.267±0.03f	35
8	500	0.244±0.05g	31
9	1000	0.229±0.01h	28
10	2000	0.209±0.02i	24

Table 14: Against FMDV the Antiviral activity of *O. europaea* stem aqueous extract.

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.215±0.04fg	25
2	7.8	0.276±0.03d	37
3	15.62	0.298±0.04c	41
4	31.25	0.333±0.03b	48
5	62.5	0.349±0.05a	51
6	125	0.243±0.01e	31
7	250	0.222±0.03f	27
8	500	0.214±0.03fg	25
9	1000	0.209±0.01g	24
10	2000	0.174±0.02h	17

* In the columns different alphabets at 0.05 significant level indicate the significant difference

* S.D means Standard Deviation

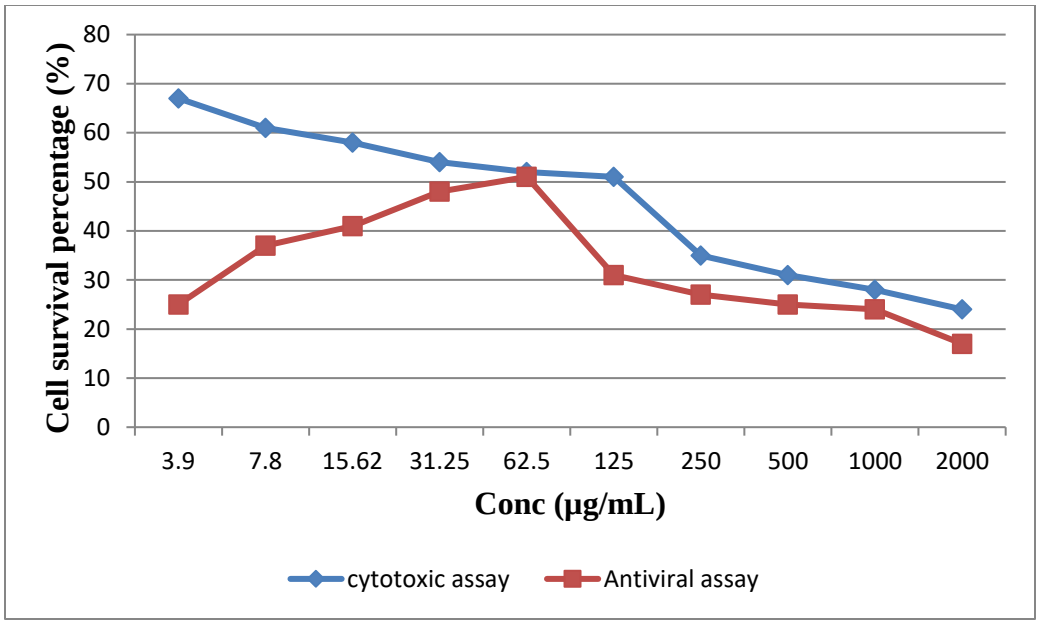

Fig7: Comparison between the antiviral and cytotoxic activity of stem aqueous extract of *O. europaea*

Table 15: for BHK-21 cells the cytotoxic activity of *O. europaea* leaf aqueous extract.

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.52±0.04a	84
2	7.8	0.495±0.02b	79
3	15.62	0.475±0.02c	75
4	31.25	0.44±0.01d	68
5	62.5	0.415±0.03e	63
6	125	0.395±0.04f	60
7	250	0.36±0.01g	53
8	500	0.351±0.02g	51
9	1000	0.335±0.02h	48
10	2000	0.314±0.04i	44

Table 16: Antiviral activity of *O. europaea* leaf aqueous extract against FMDV.

Sr. No.	Conc. used (µg/mL)	Mean O.D. ± S.D.	Cell survival percentage (%)
1	3.9	0.234±0.02f	29
2	7.8	0.263±0.03e	34
3	15.62	0.313±0.02d	44
4	31.25	0.345±0.05b	50
5	62.5	0.353±0.03a	52
6	125	0.361±0.02a	53
7	250	0.335±0.01c	48
8	500	0.312±0.02d	44
9	1000	0.256±0.01e	33
10	2000	0.217±0.04g	26

* In the columns different alphabets at 0.05 significant level indicate the significant difference
* S.D means Standard Deviation

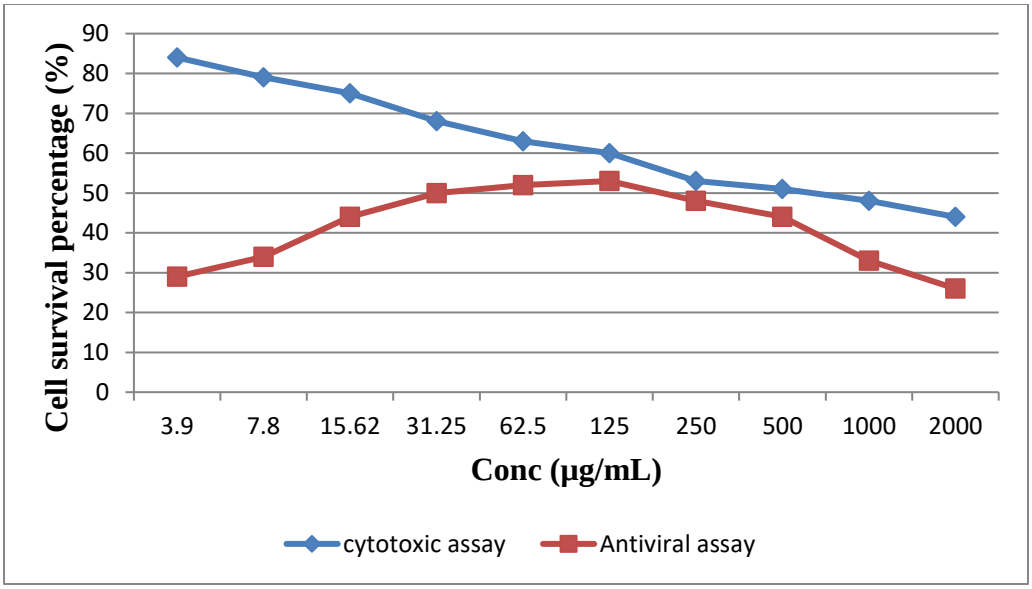

Fig 8: Comparison between the antiviral and cytotoxic activity of *O. europaea* leaf aqueous extract

4 Discussion and conclusion: It is showed by results that water, alcohol, chloroform, and n-hexane leaf and stem of *O. europaea* have various characteristics of antiviral concentration. Along with antiviral, *O. europaea* exhibited also the activity toxic, it was predicted that *O. europaea* alcohol leaf extracts had antiviral potent activity at 31.25Hg/mL-250Hg/mL concentration range along with a range of CSP was fifty-one percent (51%) to sixty-three percent (63%), that was followed by chloroforms extract activity in which percentage of cell survival was analyzed fifty-four percent (54%) or fifty-seven (57%) at concentration 31.25 Hg/mL or 62.5 Hg/mL respectively. At a concentration range of 15.62 Hg/mL-125Hg/mL *O. europaea*, n-hexane extract of leaf experienced antiviral activity. While in the extract of aqueous, the CSP was measured as fifty percent (50%) and fifty percent (50%) at a concentration of 31.25Hg/mL and 62.5Hg/mL respectively. The extracts of water and n-hexane showed the activity of cytotoxicity at a low 2000Hg/mL concentrate range, while the extract of chloroform and alcohol showed their cytotoxicity at 2000Hg/mL and 500Hg/mL.

While the order for showing the extract of effectiveness was described as

n-hexane <Water <Chloroform <Alcohol

Similarly, stem extracts of n-hexane and alcohol revealed their cytotoxicity activity at 250Hg/mL. On the other hand, extracts of aqueous and chloroform showed their cytotoxicity at 500Hg/mL and 125Hg/mL range respectively. In the same way, the stem extracts of chloroform and alcohol showed their significant antiviral activity at the concentration range of 31.25Hg/mL to 250Hg/mL (Table 6, Table10). (Younas *et al*., 2015) described with their findings that the extracts of ethanolic have the highest activity against FMDV, that extracts of ethanol have leaf concentrate of Moringa oleifera. Furthermore, the results of experiments showed that the cell concentrations were directly proportional to the cytotoxicity activity of the cell. So, the morphological change of the cell was accompanied by a concentration of extracts that were 500Hg/mL-1000HgmL and then 2000Hg/mL. However; at a concentration range of 3.9Hg/mL-250Hg/mL the cells showed their appearance as normal as well as there was no activity in cell-related to cytotoxicity. The results of this research supported by the work of (Alkan *et al.*, 2012), who started cytotoxicity activity of plants Salvia Officinalis belong to Lamiaceae family extract at the higher range of concertation. Similarly, *O. europaea* has an antiviral activity that was analyzed in this study and explained that this activity occurred because of oleuropein, that capability in vitro infectivity of disease FMDV. These findings also agreed with (Micol *et al.*, 2005) research work in which they exported antiviral activity of commercial *Olea europaea* L. and olive leaves macerate against viral hemorrhagic septicemia rhabdovirus (NHSV) as well as ,recently, the leaves of olive have constituents of calcium elenolate that have vitro capabilities against a different virus-like rhinoviruses, parainfluenza and leukemia of olive leave also reported against HIV (Salih *et al.*, 2017). Moringa oleifera has its antiviral activity due to phytochemical that is maslinic acid which called the natural antiviral agent by (Kısa *et al.*, 2018). Furthermore, this study also have similarity with work of (Kısa *et al*., 2018) who reported that maslinic acid also has anti-tumoral activity was observed in HT29 and $CaCo_3$ colon cells. Likewise, the antiviral activity of *O. europaea* against the virus occurred due to the protein that presents in this plant. Such protein that has the antiviral capability to penetrate

and bind with a virus in infected cells of mammalian and inactivate the ribosome of cells (Guller *et al.,* 2018).

Consequently, Plants considered not just a symbol of food, fuel, and shelter but they also has pharmacological characteristics due to which it used to treat various diseases related to animals and plants (Kadhim and Mohammed, 2020). So, plants that have such characteristics are termed "Medicinal plant". On the earth, almost 5 million species of plants exist in which some have antiviral qualities due to the presence of pharmacological that have antiviral potency like phenolic, alkaloids, and flavonoids (Meenakshi *et al.,* 2020). Generally, agents of Phyto-antiviral exert their action through interfering with the synthesis of amino acids which are essential for viruses, along with inactivating the viruses by preventing or shedding and assembly at the cell membrane. It penetrates directly in cells that are infected and stops the replication of the virus (Altaf *et al.,* 2020). Results showed the antiviral and cytotoxic activity of the *O. europaea* stem and leaf aqueous, alcohol, chloroform and n-hexane extracts. The results of study lineup with the previous research in which authors showed their results that the rHis-PoIFN-ω7 fusion protein was considerably expressed using Escherichia coli BL21 (DE3) strain, and the recombinant protein exhibited significant in vitro protection against FMDV, including two strains belonging to type O and A FMDV, respectively (Li *et al.,* 2019). In a number of countries, vaccines are used for the prevention of foot-and-mouth disease (FMD). However, because there is no protection immediately against FMD after vaccination due to which some antiviral agents must be developed to minimize the impacts of FMDV. The development and research on antiviral agents was being conducted to induce protection until immunological competence is produced. It was observed that combining orally the administration of the antiviral agents and complementary therapy with vaccines enhanced synergistically the antiviral activity and also preserved the survival rate and body weight in the experimental animals (Choi *et al.,* 2018). This study helpful because it is cost effective for underdeveloped countries to enhance the survival rate of animals without therapies (De Vleeschauwer *et al.,* 2017).

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