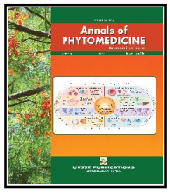
Annals of Phytomedicine 15(1): 773-786, 2026

DOI: http://dx.doi.org/10.54085/ap.2026.15.1.70

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

Evaluation of *in vitro* antiviral activity of Himalayan medicinal plants from Uttarakhand against Lumpy skin disease virus (LSDV)

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Article Info

Article history
Received 10 November 2025
Revised 21 December 2026
Accepted 22 December 2026
Published Online 30 June 2026

Keywords
Antivirals
Himalayan medicinal plants
In vitro
Lumpy skin disease virus

Abstract

Lumpy skin disease (LSD) is an arthropod-borne viral disease of cattle caused by the Lumpy skin disease virus (LSDV), characterized by nodular skin lesions, fever, lameness, mucosal lesions, nasal discharge, and lymphadenopathy. Currently, no antiviral therapy is approved, and treatment focuses primarily on supportive management. Vaccination with homologous live-attenuated and goat pox vaccines remains the primary control measure. Given the limited availability and high cost of antivirals, this study explored the antiviral potential of twenty plant extracts from nine Himalayan medicinal plants from Uttarakhand, *viz*., *Urtica dioica* L. (leaves), *Berberis aristata* DC. (leaves), *Cannabis sativa* L. (leaves), *Quercus leucotrichophora* A. Camus (leaves), *Rhododendron arboreum* Sm.(leaves and flowers), *Pinus roxburghii* Sarg. (bark), *Myrica esculenta* Buch. Ham. ex D. Don (leaves), *Zanthoxylum armatum* DC. (leaves) and *Hedychium spicatum* Sm. (roots) against LSDV. Twenty extracts were prepared using distilled water (DW) and ethanol and subjected to cytotoxicity testing on Vero cells by estimating the maximum nontoxic concentration (MNTC) and 50% cytotoxicity concentration (CC_{50}). The plant extracts were further assessed for *in vitro* antiviral activity against LSDV by measuring the reduction in virus titer ($TCID_{50}$ analysis), calculating the percentage of inhibition (PI), and estimating the EC_{50} concentration. The therapeutic index (TI) was calculated as the ratio of CC_{50} to EC_{50} values. Cytotoxicity studies revealed that the ethanolic extracts of *B. asiatica* and *Q. leucotrichophora* leaves were the safest. Potent *in vitro* antiviral activity was observed in the ethanolic extract of *Q. leucotrichophora* leaves (PI- 99%, TI- 99, $TCID_{50}$-103.551/ml), followed by the ethanolic extract of *R. arboreum* leaves (PI- 98%, TI- 59.3, $TCID_{50}$-104.551/ml), and the aqueous extract of *P. roxburghii* bark (PI- 99.36%, TI- 41.88, $TCID_{50}$-10 3.551/ml). The aqueous extracts of *C. sativa* leaves (12.55 µg/ml) and *P. roxburghii* bark (13.40 µg/ml) demonstrated high EC_{50} values, indicating their potency. The present study revealed that *Q. leucotrichophora* leaves (ethanol), *R. arboreum* leaves (ethanol), and *P. roxburghii* barks (DW) are the most effective extracts against LSDV. Overall, the present study highlighted that these Himalayan medicinal plants are promising and safe sources of antiviral agents for phytotherapeutic development. This is a novel approach to screen the activity of important Himalayan medicinal plants against Lumpy skin disease virus (LSDV), which can be further tested for similar groups of viruses of human origin.

1. Introduction

Livestock afflicted with various acute or chronic viral diseases inflict huge financial losses to the animal health sector of India. Several infectious viral diseases have been reported to date, and new ones with a high risk to both human and animal health are emerging. The risk of viral infections spreading across continents has increased in the past due to viral pandemics caused by deadly viruses (Peera and Efferth, 2012). Lumpy skin disease (LSD) is an infectious viral disease caused by the lumpy skin disease virus (LSDV) of the genus Capripoxvirus, belonging to the family Poxviridae. Viral diseases affect the economic value of animals in terms of milk and meat yield, draught power of bullocks, quality of hides, and reproductive ability. Outbreaks of lumpy skin disease (LSD) in the bovine population have resulted in significant morbidity across the nation (Sudhakar *et al.,* 2020). LSDV infects only cattle and buffalo; however, exotic and crossbred cattle are more severely affected than indigenous zebu cattle. LSDV has also been detected in wildlife species, such as Springbok and Eland in Namibia, Oryx species in South Africa and Saudi Arabia, and Guar, Mainland Serow, and Banteng in Thailand. Lumpy skin disease (LSD) is endemic in most African countries and has spread to the Middle East, Europe, and Asia since 2012, with outbreaks reported in Southeast Asia since 2019.The Morbidity rate ranges between 10 and 20%, which can be as high as 45% with a mortality rate of 1-5% under field conditions. Control of LSD relies on movement restrictions, removal of infected animals, and vaccination; without vaccination, these measures are generally ineffective (WOAH, 2021).

Treatment relies on supportive medicine to control secondary bacterial infections. Compared to the use of antibacterial agents, the use of antiviral medications in human and veterinary medicine is restricted (Dal Pozzo and Thiry, 2014). Several drugs are prescribed for viral ailments in humans. These include the use of Acyclovir against Herpes simplex virus (HSV) and cytomegalovirus (CMV); Oseltamivir and Amantadine against the influenza virus. Data on the use of antiviral chemotherapy against animal diseases are sparse.

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Ribavirin and 2'-C-methylcytidine have been found to exhibit *in vitro* anti-FMDV activity (Goris *et al.,* 2007). A novel class of imidazopyridines targeting viral RNA-dependent RNA-polymerase has been shown to have potent *in vitro* and *in vivo* activity against the classical swine fever virus (CSFV) (Vrancken *et al.,* 2008; Vrancken *et al.,* 2009). The first and most commonly used antiretroviral drug in veterinary medicine is Zidovudine, a nucleoside analog that blocks viral reverse transcriptase. This antiviral agent can effectively inhibit feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) *in vitro* and *in vivo* (Hartmann *et al.,* 1992; Dal Pozzo and Thiry, 2014). Despite these studies, the use of antiviral drugs at the field or clinical level has not yet been established. Currently, only feline interferon-ù has been licensed for use in veterinary medicine in Europe (Bracklein *et al.,* 2006). The reason behind the limited use of antiviral chemotherapy may be due to the high cost of production, poor availability, narrow spectrum of the drug, and mutations in the viral genome.

Approximately, 3000 plant species in India are known to possess therapeutic properties. The Himalayan woods in Asia are home to an abundance of medicinal plant species that support rural livelihoods by providing food, utilities, and medicine (Kala, 2005). The Himalayan region is a biodiversity hotspot, supporting over 1,748 plant species of known medicinal value. Himalayan plants such as *Rhododendron, Urtica,* and *Berberis* have been proven to have antidiabetic, antibacterial, antifungal, and antiviral properties. *Rhododendron* (flowers and leaves) is used to treat stomach aches, fever, dysentery, headaches, altitude sickness, and mental disability. It has hepatoprotective, cardioprotective, anti-inflammatory, antibacterial, analgesic, antihyperglycemic, and antihyperlipidemic properties (Paul *et al.,* 2005; Kunwar *et al.,* 2010). Approximately, 70-80% of the global population relies on medicinal plants to treat various illnesses, including viral diseases (Wang and Liu, 2014). Herbal drugs have gained importance owing to their availability and fewer side effects in patients (Edziri *et al.,* 2011). These properties can be attributed to the phytochemicals present. Phytochemicals, often referred to as secondary metabolites, are naturally present in plants. They serve as a defense against pathogenic microbes, such as bacteria, viruses, and fungi (Hasler and Blumberg, 1999). These phytochemicals can be classified as phenols, flavonoids, alkaloids, quinones, tannins, terpenes, glycosides, and polysaccharides (Sas *et al.,* 2007). They can accumulate in different plant parts, such as roots, stems, leaves, flowers, fruits, and seeds. They have been found to have a range of anti-infection, antioxidant, and anti-inflammatory properties (Kapoor *et al.,* 2017; Akram *et al.,* 2018). Many groups of phytochemicals, such as phenolics, carotenoids, terpenoids, and alkaloids, are used as antivirals (Rex *et al.,* 2018; Younas *et al.,* 2018). The viral envelope and replication mechanisms are fundamentally different from those of bacteria; therefore, antibiotics are ineffective against viral infections (Wabale, 2014). Antiviral medications restrict the growth of target pathogens, shorten the duration of infection, and are frequently used to treat viral infections in humans. However, antiviral therapy has not been successful in animals. Owing to the high rate of mutation in viruses and evolution of resistant strains, new, less hazardous antiviral medications are continuously required (Wang and Liu, 2014). Given these unsatisfactory and constrained therapeutic options, the need for novel antiviral therapies has become evident. Therefore, it is crucial to continuously research and produce novel antiviral substances. Selvaraj *et al.* (2024) revealed the significance of several phytoconstituents in the effective management of Monkeypox virus infection, which is an Orthopoxvirus. The past few decades have seen an increased interest in the quest for pharmacological compounds of plant origin that are effective against animal viruses, causing high mortality rates and significant economic losses. Herbal therapies have a significant role in this area because of the presence of different secondary plant metabolites with great therapeutic potential (Strasfeld and Chou, 2010). Residents hold strong traditional beliefs about the potency of ethnomedicinal plants used for treating illnesses. Compared to modern allopathic medications, traditional healers believe that ethnomedicinal plants are more effective and have fewer adverse effects. Most research on medicinal plant usage has been conducted in India. Plant extracts have been evaluated for their beneficial properties. Compared to the research on the antibacterial, antioxidant, anti-inflammatory, and anticancer effects of medicinal plants, plant-derived antiviral compounds have not been sufficiently studied. The Himalayan flora has not yet been fully explored against various human or animal viruses. Despite the increasing interest, antiviral studies on Himalayan medicinal plants remain limited, particularly against animal viruses such as LSDV. Scientific innovations can lead to drug design and development, where natural products, including herbal medicines, are well documented and can be translated into potent phototherapeutics (Vasanthkumar *et al.,* 2024). Addressing this gap may aid in the identification of natural antiviral agents suitable for improving animal health. There is a need to fill this research gap so that we can take advantage of the vast resources available in nature. This will help improve animal and public health. The use of antivirals in animal treatment is still in its infancy. Several antivirals used for treatment have been extrapolated from their use in human medicine. Therefore, the present study aimed to evaluate the *in vitro* antiviral activity of selected Uttarakhand Himalayan plant extracts against LSDV in the Vero cell line.

2. Materials and Methods

2.1 Collection of plant material, identification, and extraction

Plant materials from nine Himalayan medicinal plants, *viz., Rhododendron arboreum* Sm. *(*leaves and flowers), *Cannabis sativa* L. (leaves), *Berberis aristata* DC. (leaves), *Myrica esculenta* Buch. Ham. ex D. Don (leaves), *Pinus roxburghii* Sarg. (bark), *Zanthoxylum armatum* DC. (leaves), *Quercus leucotrichophora* A. Camus (leaves), *Hedychium spicatum* Sm. (roots) and *Urtica dioica* L. (leaves) were collected from Mukteswar, Nainital, Uttarakhand. The collected plant images are shown in Figure 1. The specimens are maintained at Institute with Herbarium No. UDL-1/IVRI, BAL-2/IVRI, CSL-3/IVRI, QLL-4/IVRI, RAF-5/IVRI, RAL-6/IVRI PRB-7/IVRI, MEL-8/IVRI, ZAL-9/IVRI and HSR-10/IVRI. Plant materials (leaves, bark, flowers, and rhizomes) were authenticated by Dr. Nidhi Sharma, Scientist, IVRI, Mukteswar. The materials were washed, shade dried, powdered, and stored for extraction. Extraction was performed using the Soxhlet method at 40-41°C for 4-6 h using distilled water and ethanol as solvents. The extracts were vacuum evaporated for further analysis. The extracts were dried at 41°C for further analyses.

2.2 *In vitro* cytotoxicity assay

2.2.1 Maximum non-toxic concentration (MNTC)

The MNTC was performed according to the protocol described by Gonçalves *et al.* (2005). Different concentrations of the plant extracts

in EMEM containing 2% FBS (2000 µl/ml, 1000 µl/ml, 500 µl/ml, 200 µl/ml, 100 µl/ml, 50 µl/ml, 25 µl/ml, and 12.5 µl/ml) were added in triplicate to confluent Vero cell monolayers grown in 96-well plates and incubated at 37°C and 5% CO_2 for 3-4 days. Cells were observed for morphological changes every 24 h under a microscope. The highest concentration of the extracts, which showed no appreciable cellular changes, was considered as MNTC (µl/ml). The MNTC concentrations were used for further studies on antiviral activity.

2.2.2 Determination of 50% cytotoxic concentration (CC_{50})-colorimetric MTT assay

The MTT assay was performed as described by Mosmann (1983). To confluent Vero cell monolayers grown in a 96-well plate, serial dilutions (2000 µl/ml, 1000 µl/ml, 500 µl/ml, 200 µl/ml, 100 µl/ml, 50 µl/ml, 25 µl/ml, and 12.5 µl/ml) of plant extracts in EMEM were added. The following sets of wells were maintained in each plate:

1. Sample wells - Vero cells + 50 µl plant extract + 50 µl media containing 2% FBS
2. Control wells - Vero cells + 100 µl media containing 2% FBS
3. Blank wells -100 µl media containing 2% FBS

The plates were incubated at 37°C at 5% CO_2 for 72 h. After 72 h, 20 il of MTT (2 mg/ml) was then added to each well, and plates were incubated at 37°C at 5% CO_2 for 3 h. MTT was discarded from the wells, and 100 µl of DMSO was added to each well. The plates were shaken gently. OD was measured at 540 nm using ELISA plate reader. The percentage of viable cells was calculated as follows:

$$\% \text{ cell viability} = \left[\frac{\text{Absorbance of sample - Absorbance of blank}}{\text{Absorbance of control - Absorbance of blank}}\right] \times 100$$

A. *Rhododendron arboreum* Sm. (Leaves)

B. *Cannabis sativa* L. (Leaves)

C. *Berberis aristata* DC. (Leaves)

D. *Myrica esculenta* Buch. Ham. ex D. Don (Leaves)

E. *Rhododendron arboreum* Sm. (Leaves)

F. *Pinus roxburghii* Sarg. (Bark)

G. *Zanthoxylum armatum* DC. (Leaves)

H. *Quercus leucotrichophora* A. Camus (Leaves)

I. *Hedychium spicatum* Sm. (Roots)

J. *Urtica dioica* L. (Leaves)

Figure 1: Collected Himalayan medicinal plants and their parts.

2.3 Viral inhibition assays

2.3.1 Cytopathic effect (CPE) reduction assay

Determination of antiviral activity of plant extracts by measurement of reduction of virus titre ($TCID_{50}$ analysis) was performed as per the protocol elucidated by Gonçalves *et al.* (2005). To confluent Vero cell monolayers grown in a 96-well plate, plant extracts at their MNTCs were added. This was followed by the addition of serial dilutions of LSDV to the treated cells, and untreated controls. The cells were incubated at 37°C at 5% CO_2 for 6 days duration. After 24 h, extracts and virus were discarded from the wells, and fresh EMEM enriched with FBS was replenished. Subsequent changes of media were done every alternate day. On the 6th day post infection, $TCID_{50}$ was calculated by Reed and Muench method (1938). Antiviral activity of the plant extract was expressed as percentage of inhibition (PI) using antilogarithm values of $TCID_{50}$, as follows:

$$\text{Percentage of inhibition} = \left[1 - \frac{\text{T antilog}}{\text{C antilog}}\right] \times 100$$

2.3.2 50% effective concentration (EC_{50})

EC_{50} was determined as per the protocol elucidated by Serkedjieva and Alan (1998). To confluent Vero cell monolayers grown in a 96-well plate, serial dilutions of each extract (2000 µl/ml, 1000 µl/ml, 500 µl/ml, 200 µl/ml, 100 µl/ml, 50 µl/ml, 25 µl/ml and 12.5 µl/ml) in growth media were added. After that 100 $TCID_{50}$ LSDV was then added to each well. Experiment contains infected-untreated and uninfected-treated as controls. Virus CPE was recorded after 72h. EC_{50} was calculated for all plant extracts based on visual CPE. Virus specific CPE based on cellular morphology was recorded in comparison to the virus control after 72 h.

Therapeutic index (TI) was calculated as ratio of CC_{50} to EC_{50}:

$$\text{Therapeutic index (TI)} = \left[\frac{CC_{50}}{EC_{50}}\right]$$

2.4 Statistical analysis

All the assays were done in triplicate. Statistical analyses using ANOVA were performed by the standard method described and $p<0.05$ was considered statistically significant by Snedecor and Cochran (1994).

3. Results

3.1 Cytotoxicity assays

3.1.1 Maximum non-toxic concentration (MNTC)

The results of Maximum non-toxic concentrations (MNTC) of different extracts are shown in Table 1. The highest concentration of the extracts (µg/ml), which showed no appreciable cellular changes, was considered as the MNTC (Figures 2 and 3). Among the DW extracts, the highest MNTC was observed in *U. dioica* leaves, indicating the least cytotoxicity and highest safety. This was followed by *R. arboreum* flowers, *B. asiatica* leaves, and *Z. armatum* leaves. In contrast, the lowest MNTC was found in *C. sativa* leaves and *P. roxburghii* bark, suggesting higher cytotoxic potential. In ethanol-based extracts, the highest MNTC was observed in *B. asiatica* leaves

and *Q. leucotrichophora* leaves, with lowest MNTC in *R. arboreum* leaves and *M. esculenta* leaves.

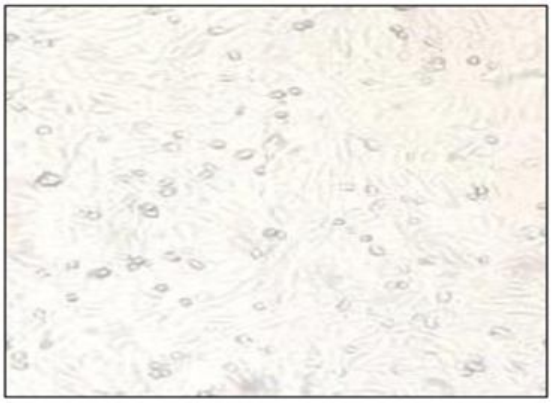
3.1.2 Half maximal cytotoxic concentration (CC_{50})

The CC_{50} values of plant extracts were evaluated using the MTT assay (Figure 4 and 5). The results are shown in Table 2. Among extracts of DW, the highest CC_{50} values were observed in *Z. armatum* leaves *U. dioica* leaves (Figure 3), and *H. spicatum* rhizome indicating the least cytotoxicity. The lowest CC_{50} value in DW extracts was recorded for *C. sativa* leaves, showing higher cytotoxic potential. In ethanolic extracts, the highest CC_{50} values were shown in *B. asiatica* leaves *Q. Leucotrichophora* leaves and *H. spicatum* rhizome with significantly lower cytotoxicity in ethanolic extracts than their DW extracts. The lowest CC_{50} was observed in the ethanolic extract of *P. roxburghii* bark. The overall highest CC_{50} among all samples was found in the ethanolic extract of *B. asiatica* leaves.

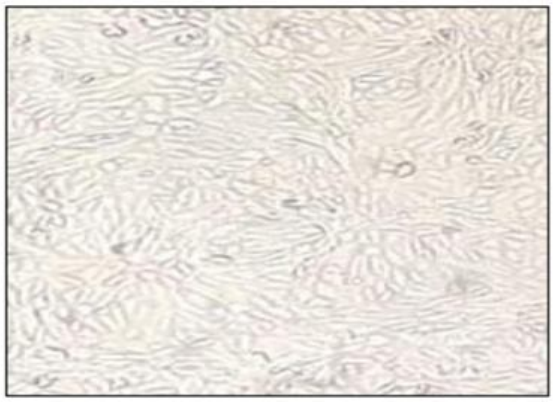
Table 1: Maximum nontoxic concentrations (MNTC) of different plant extracts

S. No.	Name of plants	Parts used	Solvent	MNTC (µg/ml)
1.	*H. spicatum*	Rhizome	DW	200
			ETH	200
2.	*U. dioica*	Leaves	DW	1000
			ETH	100
3.	*R. arboreum*	Flowers	DW	500
			ETH	100
4.	*R. arboreum*	Leaves	DW	200
			ETH	25
5.	*B. asiatica*	Leaves	DW	500
			ETH	500
6.	*M. esculenta*	Leaves	DW	100
			ETH	25
7.	*C. sativa*	Leaves	DW	25
			ETH	50
8.	*P. roxburghii*	Bark	DW	25
			ETH	100
9.	*Q. leucotrichophora*	Leaves	DW	100
			ETH	500
10.	*Z. armatum*	Leaves	DW water	500
			ETH	200

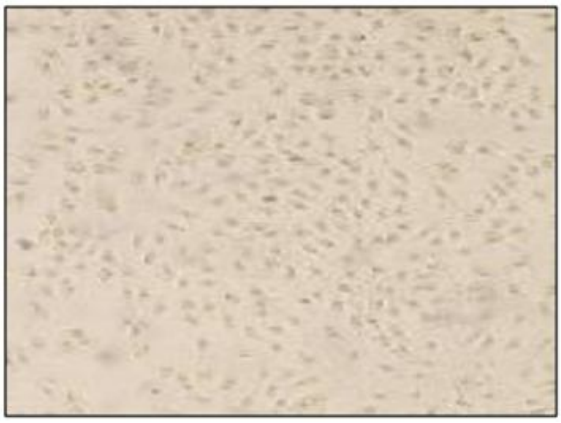
DW- Distilled water; ETH- Ethanol



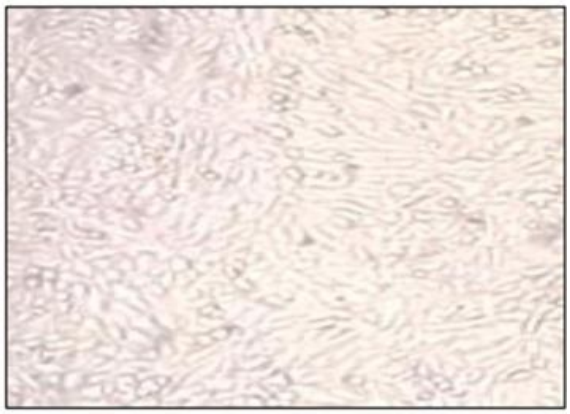
A. *H. spicatum* (Ethanol extract)- 500 µg/ml showing cytotoxicity



B. *H. spicatum* (Ethanol extract)- 200 µg/ml MNTC concentration

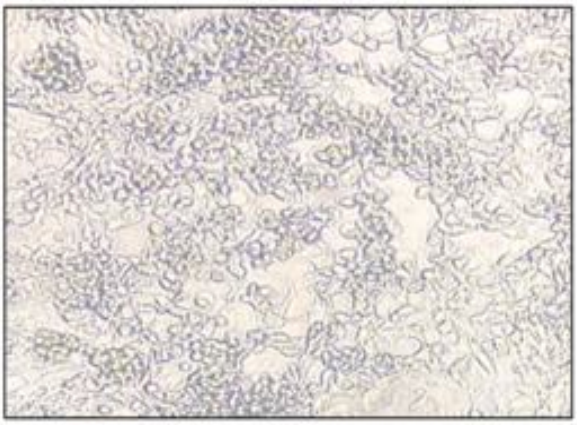


C. *Z. armatum* (Ethanol extract)- 2000 µg/ml showing cytotoxicity

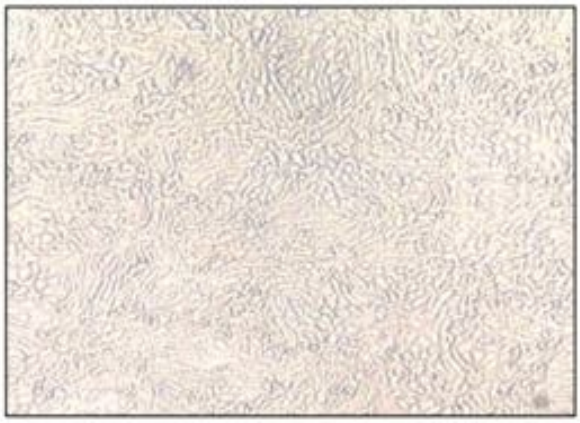


D. *Z. armatum* (Ethanol extract)- 500 µg/ml MNTC concentration

Figure 2: Cytotoxicity profile of plant extracts on Vero cells. A and C: Cytopathic effects of selected plant extracts on Vero cells at corresponding extract concentrations B and D: Absence of visible CPE at respective MNTCs.



(A)



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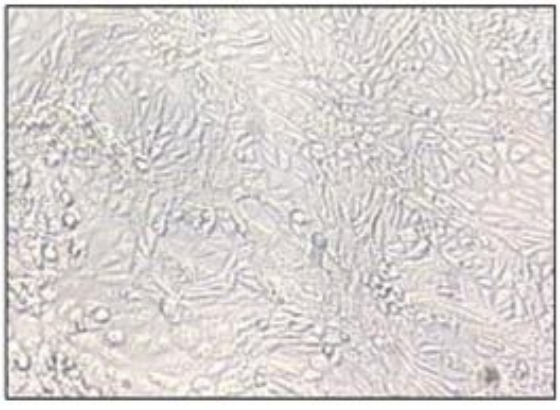

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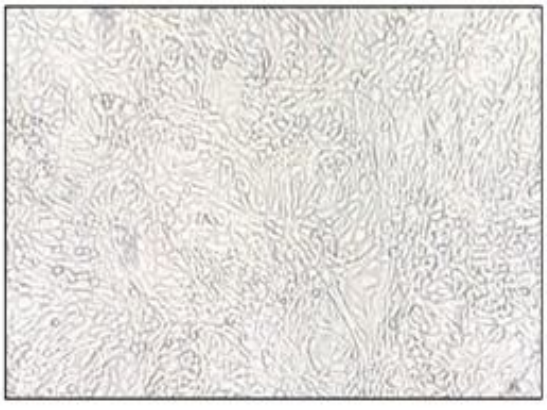


(D)

Figure 3: Comparison of Cytopathic effects between virus control and cells treated with plant extracts at MNTCs. (A) *C. sativa* (ethanolic extract) - visible CPE similar to virus control, (B) *P. roxburghii* (DW extract) -showing inhibition of CPE (C) Virus control (D) Cell control.

Table 2: Comparative analysis of CC_{50} based on MTT assay of plant extracts

S. No.	Name of plants	Parts used	Solvent	CC_{50} (µg/ml) Mean ± SEM
1.	*H. spicatum*	Rhizome	DW	1518.1 ± 1.43
			ETH	2063.1 ± 1.99
2.	*U. dioica*	Leaves	DW	1782.9 ± 2.94
			ETH	587.52 ± 4.13
3.	*R. arboreum*	Flowers	DW	1450.8 ± 3.84
			ETH	725.9 ± 0.56
4.	*R. arboreum*	Leaves	DW	1252.6 ± 2.15
			ETH	2010.0 ± 1.57
5.	*B. asiatica*	Leaves	DW	1026.4 ± 4.33
			ETH	3013.1 ± 9.31
6.	*M. esculenta*	Leaves	DW	429.2 ± 1.88
			ETH	470.8 ± 1.72
7.	*C. sativa*	Leaves	DW	290.4 ± 0.57
			ETH	687.9 ± 1.16
8.	*P. roxburghii*	Bark	DW	543.1 ± 1.2
			ETH	451.3 ± 1.82
9.	*Q. leucotrichophora*	Leaves	DW	993.1 ± 3.28
			ETH	2718.8 ± 1.55
10.	*Z. armatum*	Leaves	DW	2904.9 ± 5.59
			ETH	842.2 ± 1.22

DW- Distilled water; ETH- Ethanol

Data are presented as mean ± SEM and paired t-test were used to evaluate the differences between solvent for each plant

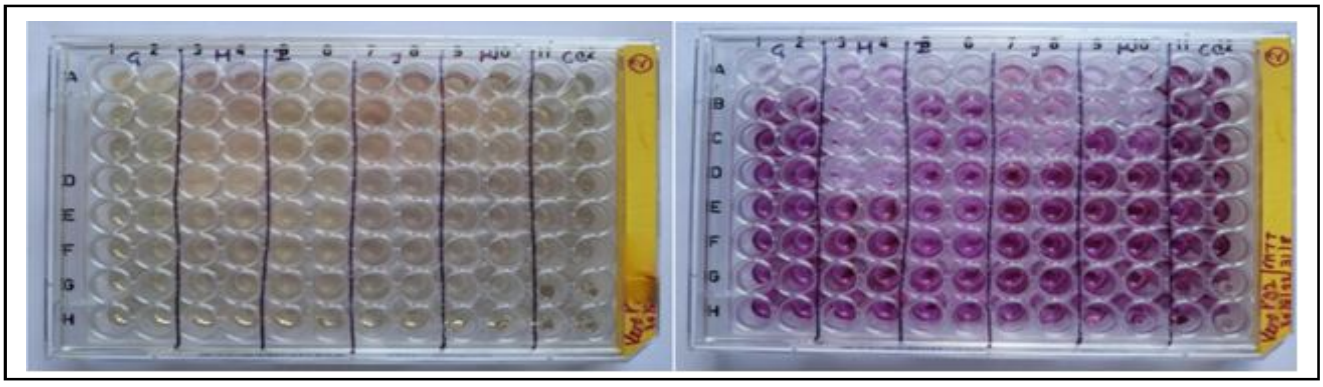


Figure 4: MTT assay for the determination of cell viability (CC_{50} evaluation).

3.2 Viral inhibition assays

3.2.1 Cytopathic effect (CPE) reduction assay

The effect of the antiviral activity of different plant extracts was evaluated by the cytopathic effect (CPE) reduction assay. Reduction of virus titre was evaluated by $TCID_{50}$ analysis, and antiviral activity of the plant extract was expressed as percentage of inhibition (PI) of virus growth. The results of the $TCID_{50}$ of extracts and cell control are shown in Table 3. Among the plant extracts, *P. roxburghii* bark exhibited the most potent antiviral activity in both distilled water and ethanol extracts, demonstrating the $10^{3.551}$ $TCID_{50}$/ml. The ethanolic extract of *Q. leucotrichophora* leaves showed similar activity with a $10^{3.551}$ $TCID_{50}$/ml value. Ethanolic extract of *M. esculenta* leaves and *B. asiatica* leaves showed relatively low $TCID_{50}$/ml values ($10^{3.801}$/ml), supporting their efficacy. The percentage inhibition activity reflects the relative reduction in viral infection when compared to the untreated virus control, demonstrating the antiviral efficacy of the plant extracts. A higher percentage indicates better antiviral activity. *P. roxburghii* bark showed the highest inhibition (99.36%) in both ethanol and aqueous extracts, indicating it is the most effective antiviral agent among the tested plant extracts.

3.2.2 Half maximal effective concentration (EC_{50})

Half maximal effective concentration (EC_{50}) of different extracts is presented in Table 4 and Figure 6. Among the aqueous (DW) extracts, *C. sativa* leaves exhibited the lowest EC_{50} value of 12.55 ± 0.55 µg/ml, indicating the highest activity, followed closely by *P. roxburghii* bark and *M. esculenta* leaves. In contrast, the highest EC_{50} values in aqueous extracts were recorded for *U. dioica* leaves, *B. asiatica* leaves, and *Z. armatum* leaves at suggesting comparatively lower bioactivity of these water-based extracts. In the ethanol extracts, a distinct pattern was observed where solvent polarity appeared to significantly influence extract efficacy the lowest.

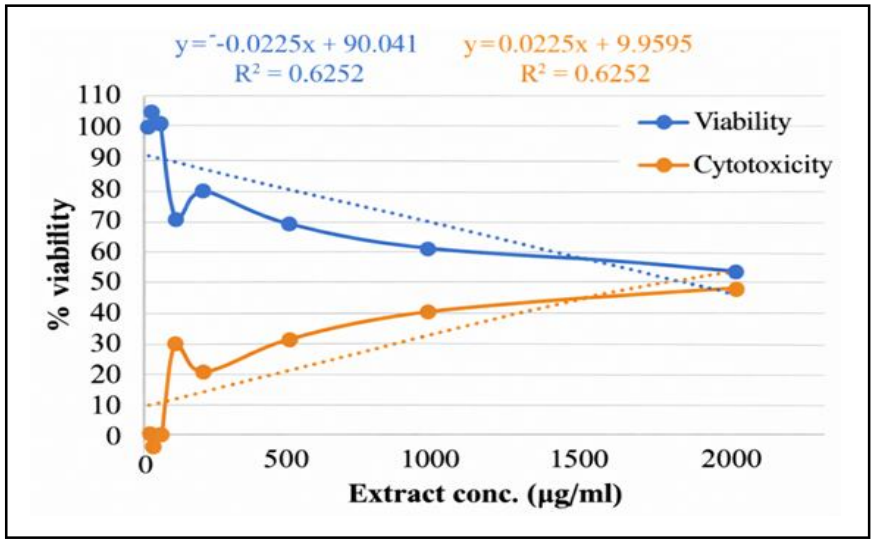


Figure 5: 50% Cytotoxicity concentration of *U. dioca* leaves (µg/ml) using MTT assay.

Table 3: $TCID_{50}$/ml and percentage of inhibition of antiviral activity of different plant extracts

S. No.	Name of plants	Parts used	Solvent	Extract ($TCID_{50}$/ml)	Control ($TCID_{50}$/ml)	Percentage of inhibition (%)
1.	*H. spicatum*	Rhizome	DW	$10^{4.801}$	$10^{5.750}$	88.78
			ETH	$10^{4.551}$	$10^{5.750}$	93.676
2.	*U. dioica*	Leaves	DW	$10^{4.551}$	$10^{5.750}$	93.676
			ETH	$10^{4.801}$	$10^{5.750}$	88.78
3.	*R. arboreum*	Flowers	DW	$10^{4.551}$	$10^{5.750}$	93.676
			ETH	$10^{4.551}$	$10^{5.750}$	93.676
4.	*R. arboreum*	Leaves	DW	$10^{4.551}$	$10^{5.750}$	98.000
			ETH	$10^{4.551}$	$10^{5.750}$	98.000
5.	*B. asiatica*	Leaves	DW	$10^{4.551}$	$10^{5.750}$	36.759
			ETH	$10^{3.801}$	$10^{5.750}$	98.875
6.	*M. esculenta*	Leaves	DW	$10^{5.351}$	$10^{5.750}$	80.001
			ETH	$10^{3.801}$	$10^{5.750}$	98.875
7.	*C. sativa*	Leaves	DW	$10^{4.801}$	$10^{5.750}$	88.78
			ETH	$10^{5.551}$	$10^{5.750}$	36.759
8.	*P. roxburghii*	Bark	DW	$10^{3.551}$	$10^{5.750}$	99.368
			ETH	$10^{3.551}$	$10^{5.750}$	99.368
9.	*Q. leucotrichophora*	Leaves	DW	$10^{3.801}$	$10^{5.750}$	98.875
			ETH	$10^{3.551}$	$10^{5.750}$	99.368
10.	*Z. armatum*	Leaves	DW	$10^{4.551}$	$10^{5.750}$	93.676
			ETH	$10^{4.801}$	$10^{5.750}$	88.78

DW- Distilled water; ETH- Ethanol

EC_{50} values were observed in *M. esculenta* leaves followed by *R. arboreum* leaves and *Q. leucotrichophora* leaves indicating that ethanol extraction enhanced the potency of these species. Conversely, the highest EC_{50} values were obtained for *P. roxburghii* bark, *H. spicatum* rhizome, and *Z. armatum* leaves. The aqueous extract of *C. sativa* leaves had the lowest EC_{50} value (12.55 µg/ml) among all the extracts. Similarly, aqueous extract of *P. roxburghii* bark had a low EC_{50} of 13.40 ìg/ml. Among ethanolic extracts, the most potent extracts with the lowest EC_{50} were noticed in *M. esculenta* leaves. Among aqueous extracts, *U. dioica* leaves showed the lowest antiviral activity, demonstrating the highest EC_{50} values against LSDV in Vero cells.

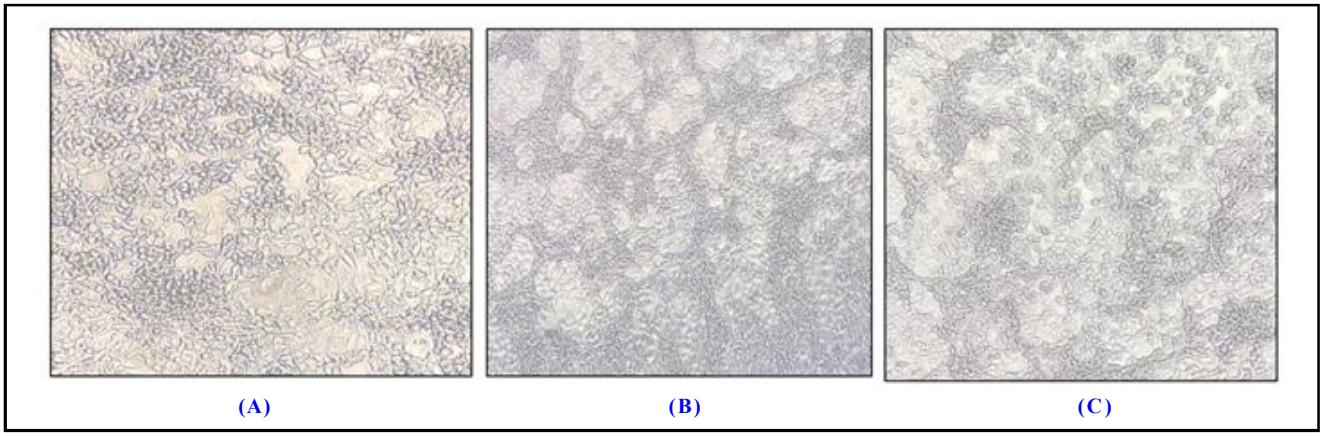
Table 4: Comparative analysis of EC_{50} based on CPE of plant extracts

S. No.	Name of plants	Parts used	Solvent	EC_{50} (µg/ml) Mean ± SEM
1.	*H.spicatum*	Rhizome	DW	111.67 ± 1.10
			ETH	123.05 ± 0.32
2.	*U.dioica*	Leaves	DW	1179.6 ± 2.15
			ETH	78.03 ± 0.20
3.	*R. arboreum*	Flowers	DW	261.02 ± 0.04
			ETH	72.68 ± 0.54
4.	*R. arboreum*	Leaves	DW	134.5 ± 0.50
			ETH	33.85 ± 0.11
5.	*B. asiatica*	Leaves	DW	1103.9 ± 2.91
			ETH	390.05 ± 0.59
6.	*M. esculenta*	Leaves	DW	62.34 ± 1.12
			ETH	15.53 ± 0.40
7.	*C. sativa*	Leaves	DW	12.55 ± 0.55
			ETH	48.46 ± 0.32
8.	*P.roxburghii*	Bark	DW	13.40 ± 0.29
			ETH	220.42 ± 0.30
9.	*Q. leucotrichophora*	Leaves	DW	64.59 ± 0.89
			ETH	27.63 ± 0.31
10.	*Z. armatum*	Leaves	DW	503.81 ± 1.08
			ETH	337.29 ± 0.58

DW- Distilled water; ETH- Ethanol

Data are presented as mean ± SEM and paired t-test were used to evaluate the differences between solvent for each plant.

The therapeutic index (TI), calculated as the ratio of cytotoxic concentration (CC_{50}) to effective concentration (EC_{50}), serves as an important indicator of the safety and efficacy of bioactive compounds. Among the ethanolic extracts, *Q. leucotrichophora* leaves showed the highest therapeutic index followed by *R. arboreum* leaves, and *M. esculenta* leaves. In the case of aqueous extracts, *P. roxburghii* (bark) recorded the highest therapeutic index followed by *C. sativa* leaves and *Q. leucotrichophora* leaves.



(A) (B) (C)

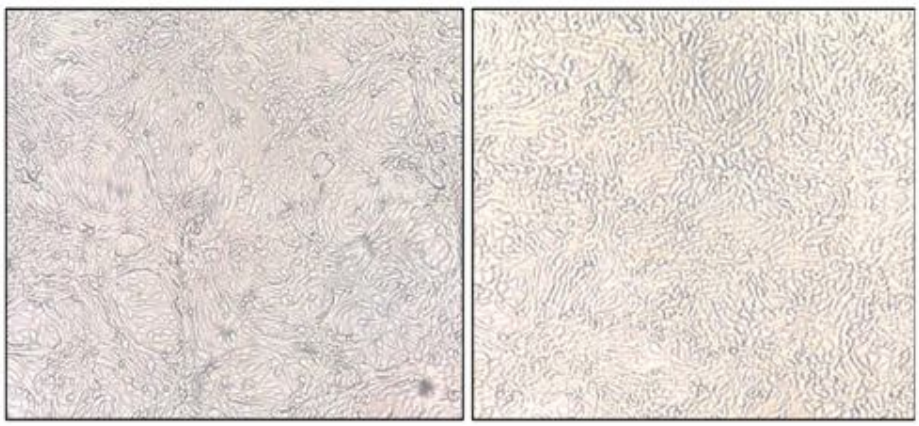


Figure 6: Comparison of different CPE scores for EC_{50} evaluation., A: CPE score 4 (76 - 100% cell death) → Virus control, B: CPE score 3 (51 - 75% cell death) → Virus infected cell treated with extract, C: CPE score 2 (25 -50% cell death) → Virus infected cell treated with extract, D: CPE score 1 (1 -25% cell death) → Virus infected cell treated with extract, E: CPE score 0 → Cell control.

4. Discussion

4.1 Cytotoxicity assays

4.1.1 Maximum non-toxic concentration (MNTC)

The determination of the MNTC is a fundamental step in evaluating the safety profile of plant extracts prior to all pharmacological studies. Assessing MNTC is crucial as it establishes the safe dosage range in which the extract can be tested for biological activity without inducing cellular damage. Cytotoxicity profiling of 20 medicinal plant extracts was performed to assess their safety in Vero cell lines. The highest MNTC (1000 µg/ml) observed for *U. dioica* (DW) suggests that this extract is relatively safe for Vero cells at the concentrations tested, offering a broad therapeutic window for subsequent antiviral testing. In contrast, the much lower MNTCs (25-50 µg/ml) for plants such as *C. sativa*, *P. roxburghii*, *R. arboreum* (leaf, ethanol), and *M. esculenta* (leaf, ethanol) indicate potential cytotoxicity at higher doses and must be handled carefully in antiviral assays. In other comparable studies, such as the *in vitro* cytotoxicity screening of indigenous medicinal plants (including Amla, Ashwagandha, Turmeric, *etc.)* on Vero cells, Bhatt *et al*. (2016) reported that no significant cytotoxicity up to 1000 µg/ml for most hydroalcoholic extracts, which closely matches our high MNTC values for certain aqueous extracts. Earlier studies on *in vitro* cytotoxicity testing of ethnomedicinal plants on Vero cells show that many extracts remained non-toxic (Jethva *et al.,* 2016). In other studies, using different plant species, the cytotoxicity on Vero cells has been even more generous. Grover *et al.* (2021) investigated *Curcuma longa* extracts and reported that more polar solvents (including ethanol) presented substantial cytotoxic potential, but still retained acceptable safety margins in Vero cells. Cytotoxicity results in this study are largely consistent with the earlier reports, particularly that the aqueous extracts display low toxicity in Vero cells. The fact that MNTCs range from 25 to 1000/ µg/ml is therefore aligned with prior evidence and reinforces the importance of solvent choice in extract preparation.

The diverse MNTCs observed in this study underscore both plant-*in vitro* cytotoxicity studies in Vero cells (Artun *et al.*, 2020; Aliyu *et al.*, 2021; Grover *et al.*, 2021). These earlier reports support the observation that many crude extracts are well tolerated by Vero cells. These data support the safe concentration ranges for subsequent antiviral screening while highlighting the necessity of cautious dose selection, particularly for extracts showing cytotoxicity at lower concentrations.

4.1.2 Half maximal cytotoxic concentration (CC_{50})

Among all distilled water extracts, *Z. armatum* leaves exhibited the highest CC_{50} (2904.9 ± 5.59 µg/ml), indicating minimal cytotoxicity. Similar low toxicity of *Z. armatum* aqueous and methanolic extracts has been previously reported, although cytotoxic effects were more pronounced in cancer cell lines where methanol-soluble saponins exerted antiproliferative activity (Alam *et al*., 2017). The significantly higher CC_{50} for the aqueous extract in the present study aligns with earlier findings that polar solvents extract fewer cytotoxic constituents from *Zanthoxylum* species than organic solvents (Alam *et al*., 2017). The aqueous extract of *U. dioica* also showed a high CC_{50} (1782.9 ± 2.94 µg/ml), consistent with studies in human fibroblasts where aqueous extracts showed negligible cytotoxicity (Asadi *et al*., 2022). In contrast, the ethanolic extract in this study exhibited a lower CC_{50} (587.52 ± 4.13 µg/ml), suggesting the extraction of more bioactive constituents with higher cytotoxic potency. This aligns with reports demonstrating that non-polar extracts (ethanolic, dichloromethane) of *U. dioica* induce apoptosis and reduce viability in cancer cells (Ergün *et al*., 2023). The difference between aqueous and ethanolic extract toxicity in the present study establishes solvent-dependent cytotoxicity profiles for *Urtica* species (Tabatabaei *et al*., 2017). *H. spicatum* rhizome extracts demonstrated moderate cytotoxicity in both the aqueous (1518.1 ± 1.43 µg/ml) and ethanolic (2063.1 ± 1.99 µg/ml) extracts. These observations are in line with earlier MTT assays where methanol extracts showed measurable but not severe cytotoxicity in HepG2 hepatocellular carcinoma cells (Choudhary and Singh, 2017).

The ethanolic extract of *B. asiatica* exhibited the overall highest CC_{50} (3013.1 ± 9.31 µg/ml), indicating very low cytotoxicity. This is consistent with observations in related species such as *Berberis vulgaris* and *Berberis lycium*, where crude extracts demonstrated minimal toxicity to non-cancerous cells while selectively inhibiting cancer cell viability at higher concentrations (Anwar *et al*., 2020). The high CC_{50} observed in the ethanolic extract therefore aligns with reports suggesting a favourable safety profile for *Berberis* extracts at moderate to high concentrations. The relatively lower CC_{50} observed for aqueous *C. sativa* leaf extract (290.4 ± 0.57 µg/ml) suggests a higher cytotoxic potential compared with other plants tested. Published studies demonstrate that both ethanolic cannabis extracts and purified cannabinoids such as cannabidiol exert dose-dependent cytotoxicity (Russo *et al*., 2011; Degenhardt *et al*., 2020; Rani *et al*., 2023). For other species evaluated in this study, including *R. arboreum*, *M. esculenta*, *P. roxburghii,* and *Q. leucotrichophora,* published data on cytotoxicity in mammalian cell lines are limited. Nonetheless, the solvent-dependent patterns, generally higher CC_{50} in DW extracts and variable effects in ethanol, are consistent with known principles of phytochemical extraction and toxicity (Alam *et al*., 2017).

4.2 Viral inhibition assays

4.2.1 Cytopathic effect (CPE) reduction assay

Viral inhibition assays are essential for evaluating the ability of plant extracts to suppress viral replication under controlled *in vitro* conditions. These assays provide a direct and quantifiable measure of antiviral efficacy by determining how effectively a test substance can prevent virus replication. The CPE reduction assay is one of the most widely employed and reliable *in vitro* methods for evaluating antiviral activity of plant extracts or therapeutic agents. Its importance lies in its ability to directly measure the inhibitory potential of a test substance on virus-induced cellular destruction. The CPE reduction assay conducted in Vero cells revealed marked differences in the antiviral potential of various plant extracts against LSDV, as reflected through $TCID_{50}$ ml values and PI. *P. roxburghii bark* extract showed strong antiviral activity, achieving the lowest viral titre of $10^{3.55}$ $TCID_{50}$ ml and 99.36% inhibition, marking it as the most potent plant extract in our study. Earlier studies on *P. roxburghii* primarily report antioxidant, antibacterial, antifungal, anti-inflammatory, and immunomodulatory effects, with little to no prior antiviral data (Kaushik *et al*., 2012). The ethanolic extract of *Q. leucotrichophora* leaves showed strong antiviral activity, with a $TCID_{50}$ of $10^{3.55}$ and high PI, comparable to *P. Roxburghii* bark. Oak species are rich in flavonoids, tannins, and phenolic compounds, which are reported to contribute to antiviral effects (Semwal *et al*., 2018). Similar antiviral activity has been observed in *Quercus brantii* against HSV. *Quercus persica* extracts also demonstrated antiviral potential linked to phenolic content (Karimi *et al*., 2013). These results highlight the unexplored antiviral potential of *Q. leucotrichophora* and warrant further study to isolate bioactive compounds (Kumar *et al*., 2020). The extracts of *M. esculenta* and *B. asiatica*, particularly in ethanol, also exhibited strong antiviral activity with $TCID_{50}$/ml values of $10^{3.801}$ and inhibition exceeding 98%, supporting earlier findings that these species possess bioactive phytochemicals such as flavonoids, tannins, and berberine with proven antiviral potential (Singh *et al*., 2018).

In contrast, the ethanolic extract of *C. sativa* leaves and the aqueous extract of *B. asiatica* leaves showed weak antiviral activity, with higher $TCID_{50}$ ($10^{5.551}$ and $10^{4.551}$ respectively) and only 36.75% inhibition. Previous studies by Iqbal and Matsabisa (2024) reported variable antiviral efficacy in *C. sativa* extracts, with more work focused on antioxidant or antimicrobial effects rather than antiviral potency. In *Berberis* species, the major alkaloid Berberine has demonstrated potent antiviral effects by reducing Influenza A replication, inhibiting Chikungunya virus during *in vitro* and *in vivo* study (Yan *et al*., 2018). Furthermore, antimicrobial studies on *B. asiatica* bark show stronger effects in methanolic extracts than in aqueous ones, highlighting that extraction solvent critically shapes efficacy (Bhandari *et al*., 2000). Notably, *R. arboreum* leaf extracts (aqueous and ethanolic) showing 98% viral inhibition align with prior reports that *R. arboreum* is rich in phenolic compounds (*e.g.,* quercetin, gallic acid, flavonoids) that have strong bioactivity (Painuli et *al*., 2018). Moreover, *in vitro* antiviral assays with *R. arboreum* petals demonstrated dose-dependent SARS-CoV-2 inhibition, implicating its polyphenols (*e.g.,* caffeoyl-quinic acids, catechin) in viral suppression (Lingwan *et al*., 2023). The overall trend of the present study, where most extracts exhibited inhibition values greater than 80%, correlates with earlier findings supporting the antiviral capacity of Himalayan medicinal plants against poxviruses and other DNA viruses. Collectively, the results confirm that several of these traditionally used medicinal plants, especially *P. roxburghii*, *Q. leucotrichophora*, and *R. arboreum*, harbour potent bioactive compounds capable of significantly inhibiting LSDV replication *in vitro*, offering promising leads for future antiviral phytotherapeutic development.

4.2.2 Half maximal effective concentration (EC_{50})

EC_{50} is the concentration of a drug that produces 50% of its maximum effect. A lower EC_{50} means the drug is more potent. Among the aqueous extracts, *C. sativa* leaves exhibited the strongest cytoprotective effect, demonstrated by the lowest EC_{50} value (12.55 µg/ml), followed closely by *P. roxburghii* bark (13.40 µg/ml), indicating the presence of highly water-soluble bioactive constituents in these species. In contrast, *U. dioica* and *B. asiatica* leaves showed extremely high EC_{50} values in DW extracts, suggesting limited antiviral activity. Ethanol extracts generally exhibited enhanced activity, with *M. esculenta* leaves showing the most potent response (15.53 µg/ml), followed by *R. arboreum* leaves and *Q. leucotrichophora* leaves, indicating improved solubility of active secondary metabolites in semi-polar solvent. These findings align with earlier studies reporting antiviral or antimicrobial performance of ethanol-based plant extracts compared to aqueous preparations, largely due to enhanced extraction of phenolics, terpenoids, and flavonoids. Similar observations have been reported in other plant-extract antiviral effect studies. *In vitro* screening of 17 Vietnamese medicinal plants showed that ethanol extracts often had stronger anti-porcine epidemic diarrhoea virus activity than aqueous extracts (Trinh *et al.,* 2021). Prior work evaluating 60 medicinal plants for antihepatitis B virus activity found that crude ethanolic extracts yielded some of the most effective IC_{50} (Arbab *et al.,* 2017). Previous research on *C. sativa* and *M. esculenta* has also documented significant antiviral and immunomodulatory properties, supporting the potent activity observed in the present study (Mahmud *et al.,* 2021). There is strong evidence which substantiate the anti-inflammatory, antioxidant, antifungal, antimicrobial, activity of cannabinoids presents in *C. sativa* leaves

(Kumar *et al.,* 2023). Important bioactive compounds like flavonoids, polyphenols, alkaloids, tannins and terpenoids have significantly reduced SARS-CoV-2 activity through inhibition of either entry of virus, its replication, and also by modulation of the immune system (Kabilan *et al.,* 2025).

4.3 Therapeutic index (TI)

The Therapeutic index (TI) values derived from CC_{50} and EC_{50} of the aqueous and ethanolic extracts of the ten medicinal plant species demonstrated clear solvent-dependent variability in both efficacy and cytotoxicity, emphasizing the pivotal role of the extraction medium in determining the pharmacological performance of plant-derived compounds. Overall, ethanolic extracts exhibited markedly higher TI values than aqueous extracts, a trend that aligns with earlier reports attributing increased therapeutic potency in ethanol-based extractions to enhanced solubility of polyphenols, flavonoids, and other bioactive constituents. Among the ethanol extracts, the highest TI observed in *Q. leucotrichophora* leaves, followed by *R. arboreum* and *M. esculenta*, indicates a broad safety margin and supports previous findings on the strong antioxidant and antiviral potential of these Himalayan species (Kumar *et al*., 2019). Conversely, the substantially lower TI values of *Z. armatum* leaves and *U. dioica* leaves in ethanol suggest comparatively weaker selectivity, paralleling earlier studies that reported limited antiviral specificity in these plants despite their known ethnomedicinal relevance (Singh *et al*., 2020). In aqueous extracts, the relatively higher TI of *P. roxburghii* bark and *C. sativa* leaves is consistent with earlier literature describing water-soluble constituents, particularly phenolics and terpenoids, as key contributors to their moderate therapeutic potential. Phytochemicals are plant-derived secondary metabolites that are therapeutically important because of their wide structural diversity, safety and potent biological activity. Phytochemicals possess antiviral activity by targeting life cycle of virus by interfering cell attachment, entry, replication, and release. It can also enhance the host immune response while reducing inflammation and oxidative stress. Their ability to act on different viral and host targets minimizes the development of drug resistance. Therefore, phytochemicals are promising and sustainable agent for antiviral drug development against emerging, re-emerging viral infections. Phytotherapeutics is a vital bridge between traditional medicine and modern antiviral drug discovery, particularly in the fight against emerging and drug-resistant viral infections. In present study, low TI values recorded for *B. asiatica leaves and U. dioica* leaves in DW reinforce previous observations that their principal bioactive alkaloids and flavonoids exhibit poor water solubility, leading to reduced pharmacological performance. Collectively, the present findings substantiate earlier research trends and affirm ethanol as a superior extraction solvent for maximizing the therapeutic potential of these medicinal plants in antiviral applications (Joshi *et al*., 2021). The current study can be further strengthen by phytochemical characterization of effective medicinal plants, focusing on mechanism of actions of compounds and it also needs *in vivo* trials for translation into field applications.

5. Conclusion

The present study assessed twenty extracts of nine Himalayan medicinal plants and showed strong species and solvent-dependent differences in *in vitro cytotoxicity* and antiviral activity. *In vitro* antiviral evaluation against LSDV revealed *Q. leucotrichophora* (ethanol), *R. arboreum* (ethanol), and *P. roxburghii* (DW) are the most effective extracts. These medicinal plants with isolation of bioactive compounds and controlled *in vivo* trials in target species can lead to development of better therapeutic alternative in management of Lumpy skin disease in animals. This phytotherapy will be cost effective and without side effects.

Conflict of interest

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

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Citation **Chhering Dikit, Amol Gurav, Amit Kumar, A. P. Madhusoodan, Asha Yadav, N. S. Kharayat and Y.P.S Malik (2026). Evaluation of *in vitro* antiviral activity of Himalayan medicinal plants from Uttarakhand against Lumpy skin disease virus (LSDV). Ann. Phytomed., 15(1):773-786, http://dx.doi.org/10.54085/ap.2026.15.1.70.**