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Ocimum sanctum: *in vitro* antiviral potential against animal viruses

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Ocimum sanctum, a well and widely used medicinal plant has been reported to be efficient for many ailments quoted in Ayurvedic literature. It has been reported to treat the various ailments due to viral infections in humans like measles, chickenpox, influenza and Smallpox. Besides, it has also shown its potential to give protection in malignancy related disorders. The present study was carried out to screen its potential against animal viruses infecting the dairy animals. Bovine Herpes Virus-type-1 (BHV-1) and Foot and Mouth disease virus (FMDV) affecting cattles and Newcastle Disease Virus (NDV) affecting poultry were screened. Cytopathic inhibition test was used to check the antiviral effect of *O. sanctum* against BHV-1 and FMDV in MDBK and BHK cell lines, respectively. Chick embryo fibroblasts were cultured to propagate NDV and tested by haemagglutination inhibition test. The maximum non-toxic dose of *Ocimum sanctum* leaves was determined in MDBK and BHK cell lines as well as in chick fibroblast by MTT assay. *Ocimum sanctum* nontoxic concentrations, 2.5 mg/mL, 5 mg/mL and 10 mg/mL were found in MDBK cell line, BHK cell line and chick fibroblast culture respectively. Concentrations lower than MNTD were used for studies. 85.3% and 98.4% protection were recorded against BHV-1 and NDV, respectively. However, no significant effect against FMD virus was observed. Thus, it can be effectively utilised in curing these viral diseases in farm animals.

Keywords: Antiviral activity, BHV-1, Holy Basil, NDV, *Ocimum tenuiflorum*, Tulsi

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Viruses are hazards to humans as well as to animals. Animals play a significant role which could be reported back to ancient civilization. They contribute a part to the world economy in many forms like in the food industry, dairy industry, agriculture, etc. Thus, there is a need for an efficient strategy for combating the diseases in animals. The antiviral drugs which are now available are synthetic or chemically synthesised and often give an unsatisfactory performance and show poor results against viral resistance coupled with the problem of viral latency[1-4].

India is a biodiversity hotspot, with various types of plants pooled in nature that show different actions on a different disease, further with the knowledge of Indian ancient literature on Ayurveda, all this could be used for the benefit of human and animal race. Ethnopharmacology, a branch of science which deals with the medicinal plants and their properties, provides an alternate approach for the discovery of antiviral agents that could be beneficial without causing any side effects. Study on approaches for use of medicinal plants in curing several diseases is continuously increasing and supported due to many positive points linked to it like immunomodulating activity, cost-effectiveness, availability, minimized or no side effects, etc[5,6]. One of the most sacred plants of India called as 'Tulsi' or 'Holy basil' [*Ocimum tenuiflorum* (synonym *Ocimum sanctum*)], is a salient part of every Indian Hindu household. It is a pool of many undiscovered elements in it which have wide use to cure several diseases. It has been found to show antiviral effects on human viruses like H9N2 virus[7], Herpes simplex virus[8], Hepatitis B virus, Adenovirus[9] and Avian influenza virus[10]. This study was designed to investigate the potential of this plant against animal viruses affecting the farm animals. Aqueous leaves extract of *Ocimum sanctum* was tested against BHV-1 and FMD viruses causing deadly diseases in cattle. With this, it was also tested against NDV virus causing infection in poultry.

Materials & Methods

Collection of plant materials

Leaves of *Ocimum sanctum* plants were collected from the garden of Veterinary University, Mathura in the months from August to December 2015 when the

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plants were well growing. Authentication of the leaves was carried out from NBRC, Lucknow. Leaves were properly cleaned by washing with distilled water and then dried in shade. Dry powder was made by crushing the leaves in the blender and kept at 4°C in airtight containers.

Preparation of aqueous extracts

Extract was made by cold extraction (aqueous) method. The extract was filtered through Whatman no. 1 filter paper, lyophilized and used for all experimental studies.

***In vitro* antiviral effect of *Ocimum sanctum* against BHV-1 and FMD virus**

Viral isolates

BHV-1 and FMD viruses were obtained from Dept. of Epidemiology, Veterinary University (DUVASU), Mathura.

Cell line

MDBK (Madine-darby Bovine kidney cells) and BHK-21 (Baby Hamster Kidney cells) maintained in Dulbecco's Minimum Essential Medium (DMEM) and Glasgow Minimum Essential Medium (GMEM) respectively were procured from Niche Area of Excellence Project, Dept. of Epidemiology, (DUVASU), Mathura.

Maximum non toxic dose (MNTD) determination

In 96 well tissue culture plate confluent monolayer of cells of MDBK and BHK cell lines were treated with 100 µL of 2-fold dilution of extract prepared at concentration of 20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 and 0.039 mg/mL in triplicates and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 3 days. The numbers of viable cells were estimated by using the MTT dye uptake assay[10]. 10 µL of MTT dye (5 mg/mL) was added to each well and plates were further incubated for 4 h at 37°C. After removal of the supernatant, the dye utilized by the viable cells was extracted with 100 µL DMSO by shaking for 15 min at room temperature. The optical density was measured at dual wavelength 560-670 nm using ELISA reader in control (without extract) as well as in extract treated wells. The maximum concentration at which there was no reduction in the optical density in experimental group as compared to control wells was regarded as maximum non-toxic dose of that extract.

BHV-I virus titration in the MDBK cell line

MDBK cells $2x10^5$/well were seeded in a 96 well plate in 0.1 mL medium and incubated at 37°C in DMEM growth media for 24 h. The supernatant was removed from each well and monolayers were infected with membrane filtered cell-free virus in 0.1 mL of DMEM containing 5% newborn calf serum. The cultures were incubated at 37°C for 5 days. The numbers of viable cells remaining were estimated by using the MTT dye uptake assay described previously. The virus titer causing 50% death was calculated from the dose-response curve and expressed as $TCID_{50}$ (50% Tissue culture infective dose).

FMD virus titration (TCID50) in BHK-21 cell line

BHK-21 cells $2x10^5$/well were seeded in a 96 well plate in 0.1 mL medium and incubated overnight at 37°C in GMEM growth media for 24 h. The supernatant was removed from each well and monolayers were infected with 10 fold dilutions of cell-free virus in 0.1 mL of GMEM containing 5% newborn calf serum. The cultures were incubated at 37°C for 5 days. The number of viable cells was assessed by MTT dye assay as described previously. The virus titer causing 50% death was calculated from the dose-response curve and expressed as $TCID_{50}$.

Antiviral assay

Non-toxic concentrations of the extract of leaves of *Ocimum sanctum* were tested for the antiviral property against BHV-I and FMD by cytopathic inhibition test in MDBK/BHK-21 cell lines. Reduction in virus-induced cytotoxicity was measured, by using the MTT dye uptake technique in extract-treated MDBK and BHK-21 cells challenged with 10 $TCID_{50}$ viruses. In brief, cells were seeded in a 96 well microtiter plate with $2x10^5$ cells/well, incubated at 37°C with 5% CO_2 for 24 h. The supernatant of the medium was removed and 10 $TCID_{50}$ challenge dose, with the different non-toxic concentration of the plant extracts in maintenance medium with 5% serum, was added in cell cultures and incubated at 37°C for 5 days. Every 24 h, observation was made microscopically using inverted microscope and the cytopathic effect was recorded. The virus suspension and dilution medium without sample were also added to the cell cultures to serve as the virus control and cell control respectively. Cell viability was evaluated by the addition of 10 µL MTT (5 mg/mL) and measuring optical density at 560-670 nm as described

previously. The percentage of protection was calculated by the following formula:

(ODT)V-(ODC)V /(ODC)M-(ODC)V X 100%

Where (ODC)M is the absorbance of control wells without virus infection whereas (ODC)V (ODT)V and correspond to absorbencies in virus-infected cells without plant extracts and with plant extracts respectively.

In vitro antiviral effect of *Ocimum sanctum* extract on ND virus

Propagation of ND virus

ND virus strain, obtained from IVRI, Izatnagar, Bareilly, was propagated in the allantoic cavity of 9-11 day old embryonated eggs and concentration of virus in the allantoic fluid was estimated by haemagglutination test (HA).

Chick embryo fibroblast (CEF) culture

Chick embryo fibroblast cultures were prepared and incubated at 37°C for 24 h for the formation of cell monolayer in 6 well culture plates at $5x10^6$ cells/mL.

Determination of maximum non-toxic dose of Ocimum sanctum extract in CEF cultures

Before estimating the antiviral activity, the maximum nontoxic dose of the plant extract was calculated on CEF culture. The extract was diluted so as to contain 50, 20, 10, 5, 2.5, 1.25 & 0.625 mg/mL of extract in the maintenance medium. Each dilution of 1 mL was inoculated to CEF cultures and incubated at 37°C in 5% CO_2. The toxic effect of each concentration was observed by inverted microscope at 12 h interval up to 48 h. Highest dilution showing any degeneration change in cell culture was considered as cytotoxic dose.

Antiviral activity of Ocimum sanctum extract

ND Virus having 0.512 HA units was added in CEF cultures with/ without extract. Three different concentrations of *Ocimum sanctum* extract less than nontoxic dose were added in cultures with ND virus. Growths of fibroblasts were monitored at different extract concentrations microscopically and HA titer in the culture supernatants of these wells were determined at different time intervals using sheep RBC's.

Results

In vitro antiviral effect of *Ocimum sanctum* against BHV-1 virus

MNTD of Ocimum sanctum in MDBK

The dose at which no degenerative changes occur in cell culture was taken as maximum nontoxic dose (MNTD). Up to the 2.5 mg/mL concentration of *Ocimum sanctum* extract, MDBK cell line did not show any toxicity. Thus, it was considered as MNTD in the MDBK cell line. The protective action of *Ocimum sanctum* extract was measured at a concentration below 2.5 mg/mL (Fig. 1).

TCID50 of BHV-1 in MDBK cell line

$TCID_{50}$ of BHV-I was determined in MDBK cell line as described in the method section and it was found at 10^{-3} virus dilution when optical density was reduced to 50% (Fig. 2).

Protection against BHV-1virus

O. sanctum was found to be very effective against BHV-1 virus causing disease in cattle. 63.7% and 85.3% inhibition were recorded by non-toxic concentrations of *O. sanctum* i.e., 1.25 and 2.5 mg/mL, respectively (Table 1).

In vitro antiviral effect of Ocimum sanctum against FMD virus

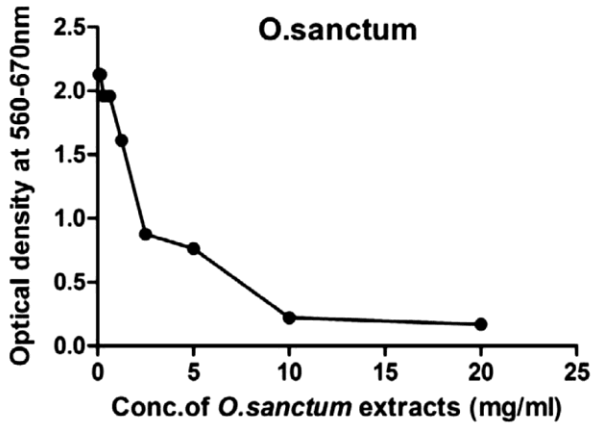

Fig. 1 — Maximum non toxic dose of *Ocimum sanctum* extract in MDBK cell line

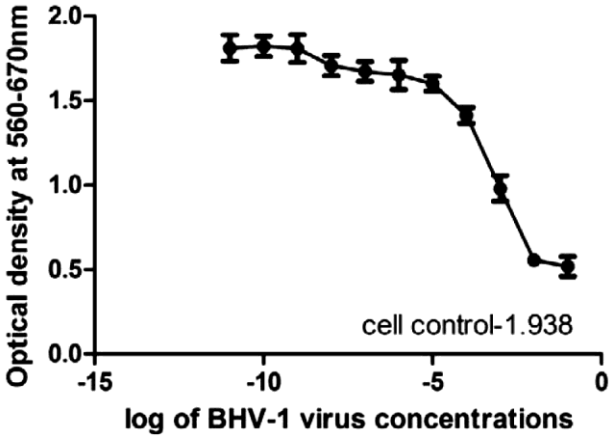

Fig. 2 — 50% Tissue Culture Infective dose of BHV-1 virus in MDBK cell line

Table 1 — % Protection against BHV-1

Culture wells with MDBK cells	OD±SEM	% Protection $(OD_T)_V$-$(OD_C)_V$/$(OD_C)_M$-$(OD_C)_V$ X 100%
Control wells without virus $(OD_C)_M$	1.75725±0.059	---
Control wells with virus $(OD_C)_V$	1.36875±0.121	--
Wells with 1.25 mg/mL *O. sanctum* extract + virus $(OD_T)_V$	1.53125±0.063	63.7%
Wells with 2.5 mg/mL *O. sanctum* extract + virus $(OD_T)_V$	1.70025±0.118	85.3%

Table 2 — % protection against FMD virus

Culture wells with MDBK cells	OD±SEM	% Protection $(OD_T)_V$-$(OD_C)_V$/$(OD_C)_M$-$(OD_C)_V$ X 100%
Control wells without virus $(OD_C)_M$	1.7095±0.042	---
Control wells with virus $(OD_C)_V$	0.220±0.028	--
Wells with 2.5 mg/mL *O. sanctum* extract + virus $(OD_T)_V$	0.35325±0.023	8.9%
Wells with 5.0 mg/mL *O. sanctum* extract + virus $(OD_T)_V$	0.53875±0.022	21.3%

MNTD of Ocimum sanctum in BHK-21 cell line

The aqueous extract of leaves of *O. sanctum* was tested for their cytotoxicity in BHK-21 cell line. The *O. sanctum* leaves showed cytotoxicity above 5.0 mg/mL in BHK-21 cell line thus the non-toxic concentrations 5.0 mg/mL and 2.5 mg/mL were used to evaluate the antiviral activity against FMD virus.

TCID $_{50}$ of FMD virus in BHK cell line

$TCID_{50}$ of FMD virus was determined in BHK cell line. FMD virus can easily be propagated in BHK cell lines. Viral inhibition of 50% in cell viability of BHK cell lines by MTT assay was found at 10^{-3} virus dilution as observed by measuring optical density at 560-670 nm. This was thus considered as $TCID_{50}$ of FMD virus (Fig. 3).

Protection against FMD virus

In contrast to BHV-1 virus, *O. sanctum* extract was not found very effective against FMD virus. As compared to control wells, 8.9% and 21.3% protection was estimated (Table 2) where $TCID_{50}$ concentration of virus was added alone. The % protection was calculated by the formula given previously in the methodology section.

Effect of *Ocimum sanctum* extracts against ND virus

The dose at which no degenerative changes occur in cell culture was taken as maximum nontoxic dose (MNTD). The MNTD for *Ocimum sanctum* was found to be 10 mg/mL. Thus, three different concentrations i.e., 10, 5 and 2.5 mg/ml of *Ocimum sanctum* were taken to test the antiviral effect. It was found that 10 mg/mL of *Ocimum sanctum* concentration inhibited the ND virus replication. The HA titre of virus in the culture supernatant was

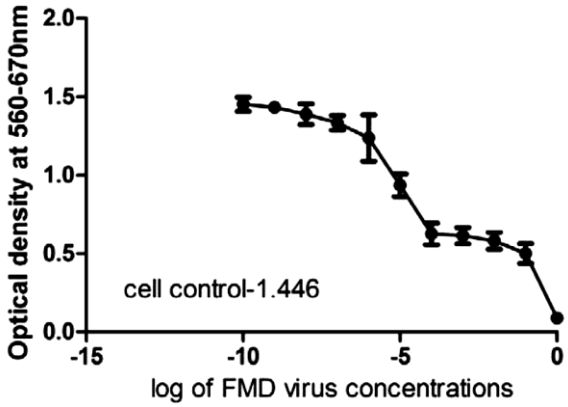

Fig. 3 — 50% Tissue Culture Infective dose of FMD virus in BHK-21 cell line

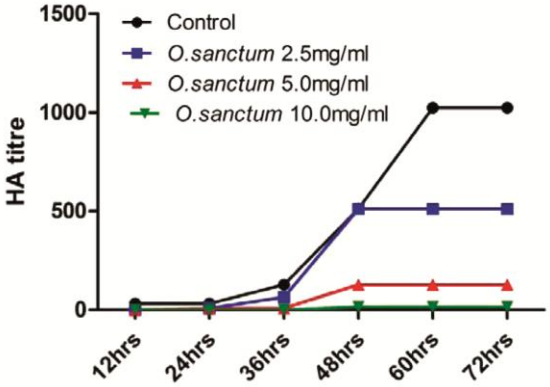

Fig. 4 — Effect of *Ocimum sanctum* extracts on ND virus in chick fibroblast cells

estimated and found to be 16 with *Ocimum sanctum* as compared to1024 titre of the control (Fig. 4).

Discussion

Agriculture and farming are the key factors for developing a sustained economy of any country. A

part of the economic growth of any country depends on the rural population. Farmers are dependent on agriculture and rearing of animals for their living. But diseases in farm animals lay a negative impact resulting in nuisance to the animal or even death sometimes, this causes loss to the individuals rearing the animal and if it turns endemic, it directly affects the economy of the country too. In animals, mass slaughter and vaccination are the two strategies used in many countries to control such spreading diseases. Slaughtering of infected animals has been successful in limiting the outbreak but low immunity animals remain very vulnerable for the spread of the diseases.

Some of the common viral diseases in animals are rabies, zika fever, encephalitis, influenza, etc. In recent years it has been observed that so many viral infections from animals are easily transferred to human beings and causes a threat to the human population. This phenomenon when a virus jumps a species is called zoonosis. Many viral diseases like bird flu, swine flu and the latest coronavirus infection have originated from animals and are now gaining attention by public health authorities. Although few antiviral drugs are available for humans in the market, but all are of synthetic origin.

Ocimum sanctum, a well-known medicinal plant in Ayurvedic literature is used as an antiviral, antifungal and antibacterial agent[11]. Besides, it has highly immunepotentiating activity[12]. Thus, the antiviral effect of this plant was investigated against FMD, IBR and ND viruses causing infections in animals. The maximum nontoxic dose of *O. sanctum* was estimated in chick fibroblast culture, MDBK cell lines and BHK-21 cell lines. Only the nontoxic doses were evaluated against different viruses. In a dose-dependent manner, the *O. sanctum* extract inhibits the growth of ND virus in chick fibroblast culture and the HA titre was reduced to 16 with 10 mg/mL *O. sanctum* extract concentration as compared to 1024 titre of the control cultures. 2.5 mg/mL and 5 mg/mL of *O. sanctum* extract also exhibit the reduction in HA titre showing antiviral potential *O. sanctum* extract against ND virus.

MNTD of *O. sanctum* in MDBK and BHK-21 cell lines was found to be 2.5 mg/mL, while the 50% Tissue culture infective doses of BHV-1 and FMD viruses were found at 10^{-3} dilution of viruses. Protection of 85.3% and 63.7% was observed by 2.5 mg/mL and 1.25 mg/mL of *O. sanctum* extract against BHV-1 virus while only 21.3% and 8.9% protection was observed against FMD virus. Choke et al also showed the virucidal activity of *O. sanctum* against the H9N2 virus[6]. Orthomyxovirus and Paramyxovirus of animals were found to be inhibited by different extracts of *O. sanctum* extract[13]. It was also found effective against multi drug-resistant *Salmonella typhi* infection[14].

Therefore, the potential uses of *O. sanctum* extract, which have multiple components and may target multiple sites, for the control of virus replication in combating a variety of viral disorders in farm animals. Since *O. sanctum* is a well known immunomodulatory, affecting various signaling cytokine molecules[15,16], will be an effective remedy during the *in vivo* infections in animals. Besides, having excellent antibacterial capabilities it can also prevent the growth of bacteria, which are opportunist as a result of suppressed immunity of the body, during viral infections. Thus, the use of it during infections also protects the animals and humans from the toxic effects of antibiotics administration. Further research should be done during the infection of these viruses in the animals and targets of the active components should be identified. This approach will lead to develop a safe and cost-effective plant-based drug which is advantageous over synthetic drugs. Bioinformatics studies, a fast high throughput screening can help in target identification of drugs.

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Conflicts of Interest

The authors declare that they have no conflicts of interest concerning this article.

Author Contributions

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