**Full Length Research Paper**

**In vitro antiviral potential of *Ocimum sanctum* leaves extract against New Castle Disease Virus of poultry**

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*Ocimum sanctum* is well known for its antimicrobial properties in Indian traditional medicinal system in Ayurveda. Different preparations of *O. sanctum* plants and parts of it have been reported throughout the world for its medicinal properties including antiviral effects. Thus in present study hot aqueous extract of *O. sanctum* leaves was used to study the antiviral activity against the New Castle Disease Virus of poultry chicken embryo fibroblast monolayer culture. Before performing the study nontoxic dose of the extract was also decided for the chicken fibroblast culture and the concentrations of 10mg/ml or less of hot aqueous extract of *O. sanctum* leaves in basal media (RPMI 1640) appeared to be non toxic. As cytopathic effects of NCD virus on chicken embryo fibroblast monolayer culture are well established so these was used to detect the antiviral activity of *O. sanctum* along with Haemaggulitation test to get an idea of viral concentration in culture. Absence of cytopathic effects in monolayer and lower the HA titer were considered as the indicative of antiviral activity of extract of *O. sanctum* leaves. The concentrations of 10mg/ml or less of hot aqueous extract of *O. sanctum* leaves prevented the cytopathic effects and growth of NCD virus in chicken fibroblast monolayer.

**Key words:** *Ocimum sanctum*, antiviral activity, New Castle Disease Virus, chicken embryo fibroblast monolayer culture

**INTRODUCTION**

Over centuries several important traditional medicinal systems such as Greek, Chinese, Tibetan, Indian, Siddha and Mediterranean have been evolved and established all over the world with the use of naturally present active principles of plants in the form of various preparations for the relief of human and veterinary diseases (Kumar et al., 2013). In Ayurveda, an Indian system of Medicine, about 2500 plants have been prized for their medicinal abilities and these are the true treasure of Indian biodiversity (Uphadhayay et al., 2013). Among these plants *O. sanctum*, distributed mainly in the tropical and subtropical region of the world, is considered to be highly scared, medicinal application in the indigenous system of medicine of many Asian, African and South American countries (Kumar et al., 2011, 2013). Since long in India the practitioner of traditional system of medicine present in villages and towns have been using *O. sanctum* for curing various ailments of microbial origin, a rational approach to this traditional medicinal practice with modern system of medicine is, however, not much available. In order to establish the scientific based therapeutic use of *O. sanctum* in modern medicine, several Indian scientists and researchers (Prakash and Gupta, 2005; Sood et al., 2006; Bhartiya et al., 2006; Goel et al., 2008; Kumar et al., 2011, 2013) have studied
the pharmacological effects of various part of this plant. However, there is little information available about its application as antiviral in poultry health and thus it appears to be the need of today as in Indian scenario poultry industry is most rapidly growing veterinary sector (Mahima et al., 2012b) and viral diseases are causing most serious losses to this industry. Among these viral diseases new castle disease that is also known as ranikhet disease is very much prevalent in Indian subcontinent and causing losses in millions every year. Henceforth, in this study in vitro antiviral activity was assessed on chicken embryo fibroblast culture with the objective to establish the antiviral properties of leaves of *O. sanctum* against New Castle Disease Virus (NCDV).

**MATERIALS AND METHODS**

**Collection of leaves of *Ocimum sanctum***: Leaves of *O. sanctum* were collected from the campus of UP Pt. Deen Dayal Upadhayay Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura and dried under shade. The leaves were verified by Department of Botany, BSA College Mathura and preserved in Department of Microbiology. Dried leaves were used for preparation hot aqueous extract.

**Viral isolate**: For antiviral effect of extract of *O. sanctum* leaves, New Castle Disease virus (NDV) was used as a challenge to the chicken embryo fibroblast culture. The virus was procured from the Department of Avian Diseases, Indian Veterinary Research Institute, Izatnagar, UP, India.

**Extraction**: Hot aqueous extract (HAE) of *O. sanctum* leaves was prepared by the method of Goel et al. (2008). In this method the 50 gram of dried powder of *O. sanctum* leaves was placed in a porous cellulose thimble. The thimble was then placed in an extraction chamber, above a collection flask containing the 750 ml solvent (triple glass distilled water). The flask was heated and the solvent was allowed to evaporate. Temperature was adjusted according to boiling temperature of the solvent (100°C). The extraction process lasted 6-8 hours and then flask containing the solvent and extract were removed. The solvent in the flask was allowed to evaporate and finally the remaining material was collected and weighed.

**Cultivation of New castle Disease virus**: The New Castle Disease virus was cultivated in embryonated chicken eggs by allantoic cavity route method as described by Cunningham (1973). The NDV was taken from deep freezer and thawed at room temperature. The eggs were inoculated by allantoic cavity route using 0.1 ml of virus inoculum per egg. Eggs were incubated at 37°C in egg incubator under moisture and examined daily by candling. Eggs dying after 2nd day were removed from incubator and allantoic fluid was collected and preserved in deep freeze (-20°C) for further use.

**Titration of New castle Disease virus**: The virus was titrated by haemagglutination test (HA test) as per the procedure advocated by Cunningham (1973). Two-fold serial dilutions of the virus was made in normal saline beginning with 1:2 dilution in 1st well through 1:1024 dilution in 10th well. 0.5 ml RBCs suspension (1%) was added in all the wells. The plate was shaken to mix the contents and incubated at room temperature for haemagglutination. Plate was examined every 15 minutes upto 1 h. for uniform haemagglutination covering the bottom of plate. Button formation was taken as negative.

**Preparation of chicken embryo fibroblast (CEF) monolayer**: The chicken embryo fibroblast monolayer was prepared as per the method described by Cunningham (1973). The embryo was taken out in petridish containing PBS. The embryo was washed with PBS and the head, appendages and viscera were removed. The embryonic tissues were cut into small pieces and washed thoroughly and transferred into a flask containing 0.25% trypsin solution (pH 7.6-7.8) in PBS for trypsinization for 1 hrs on magnetic stirrer. The contents of flasks were filtered using muslin cloth. Filtered cell suspension was centrifuged at 1500 RPM for 15 min. and supernatant was discarded. Cells pellets were resuspended with culture medium and centrifuged at 1500 RPM for 10 min. Supernatant were discarded and repeated washing was done at same RPM and time.

Cells were resuspended in medium and adjusted to1x10^7 cells/ml. 1ml of cells suspension was taken in all the wells of a six wells tissue culture plate and incubated at 37°C in an atmosphere of 80-85% humidity and 5% CO₂.

**Determination of nontoxic concentration for CEF monolayer culture**: Before conducting the antiviral properties, maximum nontoxic concentration of extract was determined in 24 hr grown monolayer CEF culture. The extract was diluted so as to contain 100, 50, 20, 10, 5, 2.5, and 1.25 mg/ml of extract in maintenance medium. 1 ml of each dilution was inoculated to CEF culture in a six wells culture plate and incubated at 37°C in the presence of 5% CO₂. The toxic effect of each concentration was observed under microscope at 12 hr intervals up to 48 hrs. Highest dilution showing any degenerative changes/CPE in cell culture was considered as cytotoxic concentration of the extract.

**Antiviral activity of *O. sanctum* extract**: Three different concentrations less than the non toxic concentration i.e. 2.5 mg/ml, 5.0 mg/ml and 10.0 mg/ml were used to determine the antiviral effect of extract of *O. sanctum* leaves. The monolayer cultures were challenged with...
Figure 1. Effect of *Ocimum sanctum* extract on New castle Disease Virus replication in chicken embryo fibroblast culture. (a) Normal growth Pattern - Control (b) Cytopathic effects produced by NCD Virus (c) Protective effect of extract on NCD virus infected cells.

NCD virus having 0.512 HA units mixed with maintenance medium. The HA titer of virus in the culture supernatant was estimated. Growth of fibroblasts was monitored and supernatant was collected at different intervals viz: 12, 24, 36, 48, 60 and 72hrs.

**RESULTS AND DISCUSSION**

In viral pathogens related problems various preparations have been attempted as ethanolic extracts (Direkbusarakom *et al.*, 1996; Parida *et al.*, 1997, Chiang *et al.*, 2005; Balasubramanian, *et al.*, 2007; Bhanuprakash *et al.*, 2008a,b), acetone extracts (Deepthi *et al.*, 2007), aqueous extracts (Parida *et al.*, 1997; Chiang *et al.*, 2005; Balasubramanian *et al.*, 2007; Bhanuprakash *et al.*, 2007, 2008) and petroleum ether, benzene, diethyl ether, chloroform, ethyl acetate, methanol and ethanol extracts (Balasubramanian *et al.*, 2007). Depending upon the type of extracts, the antiviral activity of *O. sanctum* has been assessed against many important viral agents as fish pathogenic viruses viz. Infectious hematopoietic necrosis virus (IHNV); Oncorhynchus masou virus (OMV); Infectious pancreatic necrosis virus (IPNV) (Direkbusarakom *et al.*, 1996), polio virus type-3 (Parida *et al.*, 1997), herpes viruses (HSV), adenoviruses (ADV), hepatitis B virus and RNA viruses viz. coxsackievirus B1 (CVB1), enterovirus 71 (EV71) (Chiang *et al.*, 2005), white spot syndrome virus (WSSV) in shrimp (Balasubramanian, *et al.*, 2007),) Buffalo pox virus (GTPV) (Bhanuprakash *et al.*, 2007) and infectious bovine rhinotracheitis virus (IBR) (Sharma *et al.*, 2011).

On the basis of the effects observed in all these findings hot aqueous extract of *O. sanctum* leaves were selected as absence of any other substance as ether, methanol, ethanol also interfere in virus growth. NCD virus is an important poultry pathogen with substantial economic losses to poultry industry. Initially the nontoxic dose of extract was assessed and lowest dose showing the cytopathic changes in chicken embryo fibroblast cell culture was 20.00 mg/ml in the RPMI medium. Thus all the three concentrations of 10, 5 and 2.5 mg/ml which were less than 20mg/ml were considered nontoxic concentrations and were used for anti viral activity. These doses are almost in the concurrence of the non toxic range of 22.5 to 0.175 mg/ml concentration of ethanolic extract of basil observed in VERO cells (Parida *et al.*, 1997). The antiviral effects were assessed on the basis of occurrence of changes in the CEF monolayer culture (Figure 1c) and the HA titers of NCD virus in the
Table 1. HA titer in fibroblast cell culture supernatant.

<table>
<thead>
<tr>
<th>Groups (Conc. of extract)</th>
<th>HA titer at different intervals (unit/ml)</th>
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<tbody>
<tr>
<td></td>
<td>12 h</td>
</tr>
<tr>
<td>Control</td>
<td>32</td>
</tr>
<tr>
<td>2.5mg/ml</td>
<td>0</td>
</tr>
<tr>
<td>5.0mg/ml</td>
<td>0</td>
</tr>
<tr>
<td>10mg/ml</td>
<td>0</td>
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HA titers at different intervals in fibroblast cell culture supernatant

Figure 2. HA titers at different intervals in chicken embryo fibroblast culture.

supernatant of culture. The HA titer was found significant at 16 with 10.0 mg/ml of HAE of *O. sanctum* leaves (Figure 1c) as compared to 1024 with virus control. Simultaneously it also prevented the CPE of the NCD virus (Table 1 and Figure 1b, c). The virus infection produces cytopathic effects on chicken embryo fibroblast mono-layer culture (Figure 1a, b). Any reduction in the changes or absence of CPE is supposed to be protective effects of extract. At the same time the reduced HA titer is also indicative of inhibition of viral growth. The investigation revealed that antiviral effect as evidenced on the basis of HA titer was also concentration dependent and higher concentration of extract inhibited the NCD Virus replication in corresponding higher extent (Figure 2). Sikader et al. (1998) and Kumar et al. (1997) reported the antiviral effect of different extracts on infectious bursal disease and new castle disease viruses. In present time use of herbs in poultry industry has a significant market and herbs are being added as a compulsory feed ingredient in poultry feed. The results of the study are very promising and support the use of these as feed additive to protect birds from viruses like NCD. Moreover, in the present study all the three concentrations of extract prevented the CPE with the lowest HA titer in the 10 mg/ml con-centration (Figure 2). Thus the dose of 10.0 mg/ml conc. of extract of *O. sanctum* leaves prevented the virus to multiply and can be used to get protection against NCD virus. As the role of *Ocimum sanctum* as immunomodulator has been thoroughly studied and well established (Godhwani et al., 1988; Bhartiya et al., 2006, Mahima et al., 2012b) along with antibacterial activity against common bacterial pathogens of animals (Kumar et al., 2011, 2013) thus it can be a sole source with multiple activity in poultry feed.

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