**Research Report**

**In vitro and in vivo Antiviral Potential of Hot Aqueous Extract of Ocimum sanctum and Argemone mexicana Leaves**

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**Abstract**
The present study was undertaken to explore the *in vitro* antiviral potential of hot aqueous extract (HAE) of *Ocimum sanctum* (OS) and *Argemone mexicana* (AM) leaves against Newcastle disease virus (NDV) and Infectious bursal disease virus (IBDV) in chicken embryo fibroblast (CEF) cell culture. First the nontoxic dose of HAE of OS and AM was determined in CEF. Doses of OS and AM below 20 mg/mL and 5 mg/mL respectively were found to be nontoxic to CEF in RPMI 1640 media. Anti NDV activity was determined by absence/lower cytopathic effect (CPE) in CEF and lower HA titer of cell culture supernatant. Anti IBDV activity was determined by absence/lower CPE in CEF. Besides *in vivo* antiviral effects of HAE of OS and AM against NDV and IBDV were evaluated in chicken model. 250 mg/kg body weight oral dose of HAE of OS and AM was found ideal and nontoxic in chickens and experimental chickens were fed this dose for 21 days for determination of *in vivo* antiviral effect. On 22nd day respective groups of chickens were challenged orally with ID₅₀ dose of NDV and IBDV. OS and AM fed chickens challenged with either of the virus were better protected as compared to unfed controls.

**Keywords** Antiviral; *Argemone mexicana*; *Ocimum sanctum*; Leaf extract; HAE; NDV; IBDV

**Background**

Medicinal plants have been traditionally used for different kinds of ailments including infectious diseases of bacterial and viral origin. There is an increasing need for search of new compounds with antiviral activity due to the problems of viral resistance, viral latency and recurrent infection in immune compromised patients. Since viruses are intracellular, an effective antiviral agent must prevent the replication in the infected cell without being toxic to normal cellular mechanisms (Desselberger, 1995). A number of compounds extracted from various species of plants such as tannins, flavones, alkaloids have displayed in vitro activity against numerous viruses (Vijayan et al., 2003). Studies conducted in laboratories around the world revealed that traditional medicinal plants can provide a rich source of antiviral agents (Jayati et al., 2013; Goel et al 2011; Yip et al., 1991; Taylor et al., 1996; Chiang et al., 2003).

Among these plants *O. sanctum* occupies significant place in the indigenous system of medicine of many Asian, African and South American countries. To prove the scientific basis of therapeutic use of *O. sanctum* in modern medicine, several researchers (Jayati et al., 2013; Goel et al., 2011) have explored the pharmacological effects of this plant and reported that it has antiviral activity. Besides these well-established medicinal herbs, there are some weeds like *A. mexicana* (AM) known for their toxicity. There is paucity of literature stating the antiviral effects of *A. mexicana*.

Poultry production has become a fastest growing sector to livestock economy in India as well as all over the world. To support the impetus and to grow further we should remove the constraints being faced today by the poultry industry. One of the major constraints is the viral diseases of poultry like Newcastle disease (ND) and Infectious bursal disease (IBD) viruses causing most serious losses to the poultry industry. There is little information available about OS and AM
application as antiviral in poultry health. Hence the present study was focused on to find the *in vitro* and *in vivo* antiviral potential of leaves extract of OS and AM against poultry viral pathogens such as NDV and IBDV.

1 Results

1.1 Determination of nontoxic dose of HAE of OS/AM leaves in CEF cell culture

The lowest dose of HAE of OS showing the cytopathic changes in chicken embryo fibroblast cell culture was 20 mg/mL in the RPMI 1640 medium. The doses below that i.e. 15 mg/mL, 10 mg/mL, 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, showed no CPE, hence considered nontoxic (Figure 1A~Figure 1D). For further study dose of 15 mg/mL and 12.5 mg/mL of HAE of OS were used. The lowest dose of HAE of AM showing the cytopathic changes in chicken embryo fibroblast cell culture was 5 mg/mL in the RPMI 1640 medium. The doses below that i.e. 2.5 and 1.25 mg/mL showed no CPE, hence considered nontoxic (Figure 1E~Figure 1G). For further study dose of 2 mg/mL and 1.5 mg/mL of HAE of AM were used.

![Figure 1 Determination of nontoxic dose of HAE of OS and AM leaves on CEF cells. (Observed after 72 hrs)](image)

Note: A: Normal CEF; B: OS 5 mg/mL; C: OS 15 mg/mL; D: OS 20 mg/mL; E: AM 2 mg/mL; F: AM 2.5 mg/mL; G: AM 5 mg/mL

1.2 *In vitro* antiviral activity of HAE of OS and AM against ND virus

Normal structure of cells was preserved for longer period in CEF of treated with HAE of OS/AM. The HA titer was used for determination of rate of virus multiplication in terms of HA units. It was found that the cell culture wells treated with concentration of 15 mg/mL of HAE of OS restricted the multiplication of ND virus as HA titer measured was only 32 while in untreated cell culture HA titer rose to 1024. HA titer of ND virus in cell culture supernatant treated with 2.0 mg/mL of HAE of AM leaves was 256. Present study revealed that HAE of OS and AM have antiviral activity against NDV. The details are given in the Table 1.

<table>
<thead>
<tr>
<th>Groups</th>
<th>HA titers at different intervals (unit/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24hrs</td>
</tr>
<tr>
<td>Untreated cell culture (Control)+ND Virus</td>
<td>32</td>
</tr>
<tr>
<td><em>O. sanctum</em> 12.5mg/mL+ND Virus</td>
<td>8</td>
</tr>
<tr>
<td><em>O. sanctum</em> 15 mg/mL+ND Virus</td>
<td>0</td>
</tr>
<tr>
<td><em>A. Mexicana</em> 1.5 mg/mL+ND Virus</td>
<td>64</td>
</tr>
<tr>
<td><em>A. Mexicana</em> 2.0 mg/mL+ND Virus</td>
<td>32</td>
</tr>
</tbody>
</table>
1.3 **In vitro** antiviral activity of HAE of OS and AM against IBD virus

Untreated chicken fibroblast cells inoculated with IBD virus (10^{5.4} TCID_{50}/mL) exhibited cytopathic effect after 96 hrs of post inoculation; whereas cells treated with respective concentration of extracts of either plant showed no CPE (abbreviated as NCP) except 1.5mg/mL of AM. When examined after 120 hrs of post challenge, cells treated with either concentration of HAE of OS and 2mg/mL concentration of HAE of AM also exhibited low grade CPE. However at 144 hrs post challenge cells treated with any dose of HAE of either plant revealed more CPE but less as compared to untreated group. The present study revealed that HAE of OS and AM have antiviral activity against IBDV. The details are given in the Table 2.

1.4 **In vivo** antiviral effect of HAE of OS and AM leaves

1.4.1 Determination of ID_{50} of NDV in chickens

The ID_{50} dose of ND virus was calculated to be 10^{9.5} per ml.

1.4.2 Determination of ID_{50} of IBDV in normal chickens

The ID_{50} dose of IBD virus was calculated to be 10^{3.5} per ml.

1.4.3 Observation of HAE fed chickens followed by challenge with ID_{50} dose of ND and IBD viruses

1.4.3.1 Clinical signs

Following challenge with NDV, clinical signs observed in OS and AM fed chickens were found mild as compared to control group of chicken. Out of six unfed chickens, two chickens exhibited paralysis in one leg and wings (Figure 2 B). This paralysis in leg was absent in treated chickens. Following challenge with ID_{50} dose of IBD virus groups of treated chickens with HAE of OS/AM exhibited clinical signs of low grade.

1.4.3.2 Body weight

Chicken fed with HAE of OS / AM leaves were found healthier with less loss in body weight as compared to unfed groups.

1.4.3.3 Gross lesions in visceral organs

Severe petechial hemorrhages were found in proventriculus in NDV challenged control group (Figure 3A). While chickens fed with HAE of OS and AM showed mild or less hemorrhages (Figure 3B~ Figure 3C). Hemorrhages were present in intestines of unfed chickens challenged with NDV while treated chickens showed mild lesions (Figure 3D~ Figure 3F). In IBD virus challenge, most common lesions i.e. hypertrophy of bursa of Fabricius and hemorrhages in thigh muscles were found in unfed chickens. While Chickens treated with OS and AM showed less or no effects on those parts (Figure 4A~Figure 4C).

1.4.3.4 Hematology

Unfed chickens challenged with ND/IBD virus showed increase in PCV values which greater than the pre infection value; while in case of OS fed or AM fed chickens following challenged with ND/IBD virus, PCV values were on high side but lower than that unfed (control) chickens. Unfed (Control) chickens challenged with ND virus showed leukocytosis. In AM fed group following challenge with either of virus exhibited decrease in leukocyte count. OS fed group challenged with ND/IBD exhibited heterophilia but lower to unfed as well as AM fed group. Percentage of lymphocytes in OS fed group showed little deviation while in remaining two groups there was distinct lymphocytopenia. Details are given in Table 3.

2 Discussion

For determination of **in vitro** antiviral activity of HAE of OS and AM leaves against ND and IBD viruses, the nontoxic dose was first calculated in chicken embryo fibroblast cell culture. Doses of OS below 20mg/mL in RPMI 1640 media were found to be nontoxic to CEF monolayer (Figure 1A~Figure1D). This result is similar to findings by Jayati et al (2013). Doses of AM below 5mg/mL in RPMI 1640 media were found to be nontoxic to CEF monolayer (Figure 1E~Figure 1G). Anti NDV activity of both the extracts were calculated
by HA titer in cell culture supernatant harvested at 24 hrs, 48 hrs and 72 hrs of post challenge with NDV. Both HAE of OS and AM showed inhibitory effect on multiplication of NDV. AM treated cell culture yielded higher HA titer compared to OS treated cells but lower with respect to control (Table 1). This study revealed that OS had significant antiviral property against ND virus (Table 1). This result is similar to findings by Jayati et al (2013). HAE of OS and AM treated CEF also showed normal architecture of cell structure which persisted for 72 hrs as compared to control cells. Anti IBDV activity of both the extracts were assessed by prevention of multiplication of IBDV on the basis of cell growth pattern and CPE (rounding of cells). OS and AM treated fibroblast cells maintained normal structure which persisted up to 120 hrs of incubation. While in control cells degenerative changes in CEF started to appear in 96 hrs of challenge with IBDV (Table 2).

In vivo experiment was also conducted to determine the antiviral potential of OS and AM against ND and IBD viruses. Results showed that HAE fed chickens were healthier compared to unfed (control) group. Unfed group chickens challenged with ND virus showed clinical symptoms characteristics of NDV

Figure 3 Lesions on visceral organs of unfed and fed chickens challenged with ID$_{50}$ dose of NDV

Note: A: Petechial hemorrhage in proventriculus of unfed chicken; B: Absence of hemorrhage in proventriculus of OS fed chicken; C: Mild hemorrhages in proventriculus of AM fed chicken; D: Hemorrhages in intestines of unfed chicken; E: OS treated intestine; F: AM treated intestine

Figure 4 Lesions on thigh muscle of unfed and fed chickens challenged with ID$_{50}$ dose of IBDV

Note: A: Thigh muscle of unfed chicken; B: OS treated thigh muscle; C: AM treated thigh muscle

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infection. Two chickens also showed the paralysis in one leg and wings (Figure 2B). Petechial hemorrhages were present in proventriculus in NDV challenged control group (Figure 3A). While chickens fed with HAE of OS and AM showed less hemorrhages (Figure 3B–Figure 3C). In IBD virus challenge, hypertrophy of bursa of Fabricius and hemorrhages in thigh muscles were found in challenged chickens. Chickens treated with OS and AM showed less hemorrhages on those parts (Figure 4A–Figure 4C). Hematological study was also carried out to observe the changes in blood profile. In case of unfed chicken PCV values was increased by 16.58 % and 25.25% on challenge with NDV and IBDV respectively, while in chickens fed with HAE of OS and AM following challenge PCV values were more or less static. In NDV and IBDV challenged unfed (Control) group there was a reduction in lymphocyte count by 11.98% and 17.56% respectively; but OS treated chickens challenged with NDV and IBDV showed less reduction in lymphocyte count 3.98%and 5.24% compared to pre infection values (Table 3). Lymphocyte count in AM fed chicken challenged with NDV and IBDV was markedly decreased. This study indicate that AM had suppressive effect on lymphocyte proliferation whereas OS restored the lymphocyte population (Table 3). Sadekar et al., (1998) had reported that feeding of dried leaves powder of OS @ 500 mg/chicken for 45 days orally to 15 weeks old pullets naturally infected with IBD virus exhibited immunopotentiation of both humoral and cell mediated immune responses. Our study also revealed the stimulation of both humoral and cell mediated immune responses due to OS as evidenced by increase in antibody titer and skin thickness as compared to control using S. enterica serovar Typhimurium and DNBC test (Varshney et al., 2013). Gupta and Charan (2005) studied the immunomodulatory effect of OS dried leaf powder and essential oil in infectious bursal disease virus

Table 2 Effect of HAE of OS and AM on IBDV infected CEF cell culture at different time intervals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Effect at different time intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hrs</td>
</tr>
<tr>
<td>Untreated cells culture control+IBDV Virus</td>
<td>NCP</td>
</tr>
<tr>
<td>O. sanctum 12.5 mg/mL+IBDV Virus</td>
<td>NCP</td>
</tr>
<tr>
<td>O. sanctum 15 mg/mL+IBDV Virus</td>
<td>NCP</td>
</tr>
<tr>
<td>A. Mexicana 1.5 mg/mL+IBDV Virus</td>
<td>NCP</td>
</tr>
<tr>
<td>A. Mexicana 2.0 mg/mL+IBDV Virus</td>
<td>NCP</td>
</tr>
</tbody>
</table>

Table 3 Hematology of unfed and treated chickens challenged with NDV and IBDV

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Unfed group</th>
<th>OS fed group</th>
<th>AM fed group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre infection</td>
<td>Gp (ND)</td>
<td>Gp (IBD)</td>
</tr>
<tr>
<td>PCV %</td>
<td>29.30±0.52</td>
<td>34.16±0.34</td>
<td>36.70±0.35</td>
</tr>
<tr>
<td>Hemoglobin (gm/dl)</td>
<td>9.84±0.21</td>
<td>8.32±0.17</td>
<td>9.20±0.28</td>
</tr>
<tr>
<td>TEC (million/mm$^3$)</td>
<td>2.92±0.16</td>
<td>2.80±0.42</td>
<td>2.82±0.34</td>
</tr>
<tr>
<td>TLC (th/mm$^3$)</td>
<td>22.2±0.80</td>
<td>23.10±0.84</td>
<td>21.60±0.87</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>70.6±0.34</td>
<td>62.14±0.76</td>
<td>58.20±0.83</td>
</tr>
<tr>
<td>Monocytes</td>
<td>4.56±0.32</td>
<td>5.86±0.52</td>
<td>4.60±0.65</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>3.12±0.08</td>
<td>3.74±0.26</td>
<td>3.84±0.54</td>
</tr>
<tr>
<td>Basophils</td>
<td>2.72±0.11</td>
<td>3.68±0.22</td>
<td>2.76±0.15</td>
</tr>
<tr>
<td>Heterophils</td>
<td>19.00±0.99</td>
<td>24.58±0.66</td>
<td>30.60±0.31</td>
</tr>
</tbody>
</table>

Note: All values are mean±SE of 6 chickens
(IBDV) infection in broiler chickens and observed enhanced cell mediated immune response. Gupta (2008) also found the similar results for Ocimum sanctum as antiviral and immunomodulator on chickens. Kolte et al. (1999) also observed that 2-6 weeks old broiler fed with OS in combination with leaf gall of Ficus racemosa survived natural outbreak of IBD. Our results are similar to these findings.

The present findings conclude that OS and AM leaves have antiviral activity against NDV and IBDV.

3 Material and Methods
3.1 Preparation of Hot Aqueous Extract (HAE)
HAE of OS and AM leaves was prepared using Soxhlet apparatus as per the protocol described by Goel et al. (2008). Percentage yield was 14%~16% (w/w) for OS and 16%~19% (w/w) for AM in terms of starting dried material.

3.2 Experimental birds
Standard pathogen free one day old chicks (Av. Wt 30~35 gm) were purchased from Uday hatchery, Mathura and reared at poultry farm, DUVASU, Mathura. All the birds were housed and fed under standard conditions. In each experimental group, individual bird identification was made by using wing tag. Seven day old chicks were used for the experiments. All these experiments were approved by “Institutional Animal Ethics Committee” (IAEC), and research was conducted under their guidelines. 6 birds per experimental group/control group were used.

3.3 Determination of nontoxic Dose (NTD) of hot aqueous extract (HAE) of OS and AM leaves in chickens
The non-toxic dose was determined by oral feeding of three doses 250 mg/kg, 500 mg/kg and 1000 mg/kg body weight of HAE of OS and AM leaves. For in vivo antiviral study oral feeding of 250 mg/kg HAE of OS and AM leaves was done as it was ideal (Varshney et al., 2013).

3.4 Cultivation of NDV in chickens embryonated eggs (CEE) by allantoic cavity route
ND virus obtained from the repository of Department of Microbiology and Immunology, DUVASU, Mathura was cultivated in 9~12 days old embryonated chicken eggs using allantoic cavity route (OIE, 2012). Haemagglutination (HA) test of the harvested allantoic fluid was carried out for confirmation (OIE, 2012).

3.5 Cultivation of IBD virus in chickens embryonated eggs (CEE) by CAM route
IBD virus (Georgia strain) procured from Department of Biotechnology, Indian Veterinary Research Institute (IVRI), Izatnagar was cultivated in 9~12 days old chicken embryonated eggs using chorioallantoic membrane route (CAM) (OIE, 2008). IBD virus was confirmed by agar gel immuno diffusion test (AGID) (OIE, 2008) using specific anti IBDV serum obtained from Department of Biotechnology, IVRI.

3.6 In vitro antiviral effect of HAE of OS and AM leaves against ND and IBD viruses
3.6.1 Preparation of chicken embryo fibroblast (CEF) monolayer
The chicken embryo fibroblast monolayer was prepared as per the method described by Cunningham (1973). Briefly, 9~12 days old live fertile hen eggs were selected and swabbed with 70% ethyl alcohol. The egg shells were cracked at air sac side and embryos were taken out in Petri dishes containing phosphate buffered saline (PBS). Embryos were washed with PBS and head, appendages and viscera were removed, and the embryonic tissues were cut into small pieces and washed thoroughly with RPMI 1640 medium. These small fragments were transferred to a sterilized flask containing 0.25% trypsin solution (pH 7.6~7.8). Trypsinization was done for one hour on magnetic stirrer. Contents of flasks were filtered using nytex membrane. Filtered cell suspension was centrifuged at 1500 rpm for 15 min, and supernatant was discarded. Cells pellet was washed thrice with RPMI 1640 medium. Finally cells were suspended in RPMI 1640 medium and adjusted to 1×10⁷ cells/ml. 1ml of cells suspension was taken in each well of a six wells tissue culture plate. Cells were incubated at 37°C in an atmosphere of 80%~85% humidity and 5% CO2.

3.6.2 Cultivation of ND virus in chicken embryo fibroblast
1 ml of ND virus (512 HA unit/ml) was inoculated in CEF monolayer in cell culture flask. Inoculated cell culture flasks were incubated for 2~3 days and
monolayer were examined for cytopathic effect (CPE). Monolayers were subjected to three cycles of freezing and thawing and then centrifuged at 5000 rpm for 20 minutes. Supernatant was harvested and stored at −70°C till further use. The presence of ND virus was confirmed by HA test.

3.6.3 Cultivation of IBD virus in chicken embryo fibroblast

1 mL of egg adopted IBD virus (10^5.4 TCID₅₀/ml) was inoculated in CEF monolayer in cell culture flask. Inoculated cell culture flasks were incubated for 3–4 days and monolayers were examined for cytopathic effect (CPE), if not visible blind passages (3–4 times) were given till the appearance of CPE (Hossain et al., 2006). On appearance of CPE (rounding of cells) monolayer were subjected to three cycles of freezing and thawing and then centrifuged at 5000 rpm for 20 minutes. Supernatant was harvested and stored at −70°C till further use. The presence of IBD virus was confirmed by agar gel immuno diffusion (AGID) test using specific IBD virus antiserum (Figure 5).

![Figure 5 AGID test for detection of IBDV](image)

3.6.4 Determination of nontoxic dose of HAE of OS and AM leaves in chicken embryo fibroblast (CEF) cell culture

Nontoxic dose of each plant extract was determined in 72 hrs grown uniform CEF monolayer. The extract was diluted in maintenance medium (RPMI 1640) to contain 100 mg/mL, 50 mg/mL, 20 mg/mL, 15 mg/mL, 10 mg/mL, 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL of extract of OS/AM. 1 ml of each dilution was inoculated in to respective CEF cell culture well in a six wells cell culture plate and incubated at 37°C in 5% CO₂. The effect of each dilution on normal growth condition of CEF was observed under microscope at 12 hrs intervals up to 72 hrs. Concentration causing no degenerative change/CPE in cell culture was considered as nontoxic dose of the extract(s).

3.6.5 In vitro antiviral activity of HAE of OS and AM against ND virus

ND virus having 512 HA units/ml was added in 24 hrs grown CEF monolayer with/without OS and AM extract. 15mg and 12.5mg per ml of OS leaf extract; 2 mg and 1.5 mg per mL of AM were used as nontoxic dose. Growth pattern of fibroblasts and CPE were monitored. Cell culture supernatant collected at 24 hrs, 48 hrs and 72 hrs were used for determination of HA titer taken as an index of virus multiplication. Results were compared with ND virus infected untreated CEF monolayer.

3.6.6 In vitro antiviral activity of HAE of OS and AM against IBD virus

IBD virus having 10^5.4 TCID₅₀ per ml was inoculated on to CEF cell monolayer containing nontoxic doses i.e. 15mg and 12.5mg per ml of extract of OS; 2.0mg and 1.5mg per ml of AM in maintenance medium (RPMI 1640). Growth pattern of chicken fibroblast and CPE were monitored at different time intervals like 24 hrs, 48 hrs, 72 hrs, 120 hrs and 144 hrs and compared to control well. CPE were graded like severe (+++), moderate (++), less (+) and unnoticed (−).

3.7 In vivo antiviral effect of HAE of OS and AM leaves against ND and IBD viruses

3.7.1 Determination of ID₅₀ of NDV in chickens

To determine the ID₅₀ of ND virus, 10 fold serial dilutions of the virus were prepared in sterile PBS and inoculated with 1 ml of each dilution to individual groups of six chickens by the oral route and observed for 10 days. The symptoms and clinical signs such as drooling of saliva, loss of appetite, dullness and depression, ruffled feathers, respiratory rales, gasping, sneezing, coughing, nasal discharge, paralysis of one or both legs or wings, torticollis and death were taken as characteristic of ND infection. From infected birds virus was isolated and confirmed. ID₅₀ was calculated as described by Reed and Muench (1938).

3.7.2 Determination of ID₅₀ of IBDV in chickens

To determine the ID₅₀ of IBD virus, 10-fold serial
dilutions of the virus were prepared in sterile PBS and inoculated with 1 mL of each dilution to individual groups of six chickens by the oral route and observed for 10 days. Clinical signs such as prostration, reluctance to move, ruffled feathers and frequent watery or white diarrhea, soiling of vent and tremors and death were taken as characteristics of IBD infection. From the infected birds virus was isolated and confirmed. 50% of the infected chickens were sacrificed for examination of bursa of Fabricius, thigh and breast muscle. ID<sub>50</sub> was calculated as described by Reed and Muench (1938).

3.7.3 Effects of HAE of OS/AM leaves in chickens experimentally challenged with ID<sub>50</sub> dose of ND and IBD Viruses

6 groups (GI to GVI) having 6 chickens in each were taken. Chickens of GI and GV were fed orally with 250 mg/kg body wt. HAE of OS for 21 days. Chickens of GIII and GVI were fed orally with 250 mg/kg body wt. HAE of AM leaves for 21 days respectively, while GI and GIV were unfed groups and kept as control. On 22nd day chickens of GI, GII and GIII were challenged with 10<sup>9.5</sup>/mL (ID<sub>50</sub> dose) of ND virus and chickens of GIV, GV and GVI were challenged with 10<sup>3.5</sup> (ID<sub>50</sub> dose) of IBD virus by oral route and observed twice daily up to 15 days for development of clinical signs of infection. In vivo effects of HAE of OS and AM leaves against ID50 of ND and IBD viruses were recorded by clinical signs, body weight, hematology and gross lesions in visceral organs.

3.7.4 Observation of HAE fed chickens followed by challenge with ID<sub>50</sub> dose of NDV and IBDV

3.7.4.1 Clinical signs and Body weight

Clinical signs as narrated were noted and compared in varying degree, if any with OS and AM treated chickens. Body weights of treated and untreated chickens were also noted. On the basis of severity of clinical signs developed in treated and untreated were taken as indicator of antiviral effect.

3.7.4.2 Hematology

The blood was collected after 7 days of challenge from wing vein from chicken in sterile test tubes containing EDTA @1 mg/mL of blood from OS and AM extract fed and unfed group of chickens and immediately processed for PCV, Hb, TEC, TLC and DLC as described by Feldman et al (2000).

3.7.4.3 Gross lesions in visceral organs

OS and AM fed and unfed chickens challenged with ID<sub>50</sub> of ND virus were sacrificed and examined to see the changes in liver, proventriculus and intestine. While in chickens challenged with ID<sub>50</sub> of IBD virus were sacrificed for studying the changes in breast muscle, thigh muscle and bursa.

3.7.4.4 Detection of ND/IBDV viruses in tissues

Respective virus was isolated from infected tissues of the dead/sacrificed birds and confirmed by suitable test procedure. For ND virus HA and HI test was done and for IBD virus AGID test was performed.

Authors' contribution
All the authors contributed equally for this study. All the authors read and approved the final version of the manuscript.

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