



RESEARCH ARTICLE

Evaluation of Antiviral Potential of Different Cholistan Plants against Infectious Bursal Disease and Infectious Bronchitis Virus

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ABSTRACT

The present study was conducted to determine the antiviral activity of different Cholistan plants growing in the Cholistan Desert, Pakistan. Methanolic extracts of plants were prepared by dissolving air-dried, powdered plants in methanol and later concentrated in a rotary evaporator. The concentrated extracts were dissolved in distilled water and sterilized through filtration. To assess antiviral potential plant extracts were mixed with an equal concentration of a live virus and propagated for 7-11 days in embryonated eggs with appropriate controls. After 48 hours, the allantoic fluids were harvested and *hemagglutination assay* (HA) was performed for infectious *bronchitis virus* (IBV) and indirect hemagglutination assay (IHA) for infectious *bursal disease virus* (IBDV) to determine both virus titer, as well as evaluation of the inhibitory effects of various plant extracts. Almost all selected plant extracts exhibited an antiviral effect varying from plant to plant and manifested through controlling growth of IBV except *Solanum surattense*. The methanolic extracts of two plants *Ochthochloa compressa* and *Sporobolus icolados*, showed a 100% inhibitory effect demonstrated through no viral growth determined by HA assays. Similarly, other plant extracts also showed antiviral activity in the order of HA titer at 8 in case of three plants *Haloxylon salicornicum*, *Neurada procumbens* and *Salsola baryosma*, 16 for *Achyranthes aspera*, *Haloxylon recurvum* and *Panicum antidotale*. The HA values 32 and 64, respectively, for *Oxystelma esculentum* and *Suaeda fruticosa* were revealed. In case of anti-IBDV activity, the methanolic extracts of *Achyranthes aspera*, *Haloxylon recurvum*, *Haloxylon salicornicum*, *Panicum antidotale*, *Salsola baryosma*, *Sporobolus icolados* were shown to have the optimal antiviral potential (IHA titer 0). Similarly, extract of *Neurada procumbens* kept the IHA titer at 2 and *Solanum surattense* at 4. The extracts of *Oxystelma esculentum* and *Ochthochloa compressa* were least effective in controlling multiplication of IBDV and their IHA titer was equal to virus control. Overall, our studies open up new avenues in the development of much needed antivirals, utilizing indigenous resources.

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INTRODUCTION

Medicinal plants are a rich source of natural products, especially compounds like tannins, polyphenols, proanthocyanidins sulfonamides anti-adhesives etc., exhibiting antiviral effects (Mukhtar *et al.*, 2008). Some of these compounds were tested against viruses like hepatitis C virus (HCV), and herpes simplex viruses (HSVs) (Javed

et al., 2011). The anti-HSV activity by plant derived polyphenols have been described previously (Shahat *et al.*, 2002). Droebner *et al.*, 2007 reported the antiviral effects of proanthocyanidins derived from plants against various avian influenza virus (AIV) strains either alone or in combination with other conventional antimicrobial drug(s) manifesting a synergistic effect (Silva and Fernandes, 2010). Various formulations prepared from plants have

been used in the past for treatment of viral infections. (Vijanyan *et al.*, 2004). Researchers have always been intrigued with the potentials of higher plants usage as promising sources of phyto-antivirals (Jassim and Naji 2003; Mukhtar *et al* 2008). Among several others, a novel antiviral compound Nordihydroguaiaretic acid (NDGA) isolated from creosote plant leaves, has been revealed to be lignin in nature and has received much attention as an antiviral. This compound and its derivatives have shown antiviral activity against Junin virus, the causative agent for Argentine hemorrhagic fever (AHF) (Konigheim *et al.*, 2005). Interestingly, NDGA was found to be effective against herpes simplex viruses (HSVs) both type I and II. Several other plants including *Larrea tridentata* have also shown the presence of NDGA in their extracts (Larreatat, 2000). The NDGA and its derivatives have also shown inhibitory effect on the HIV transcription process through inhibiting TAT- transactivation activity (Gnabre *et al.*, 1995). Ongoing exploratory work in the area of phyto-antivirals have led to the identification of novel antiviral agents and a wide range of active metabolites including: phyto-chemicals, furyl compounds, sulphides, proteins/polypeptides, peptides, polyphenolics, flavonoids, terpenoids, saponins, lignans, and coumarins. Various plants used herbal teas extracted essential oils showing have strong antiviral activity (Cowan, 1999; Mukhtar *et al.*, 2008).

As far as the viral infections in poultry birds are concerned it has been a major reason of economic losses to poultry flocks in the past among Asian countries including Pakistan. Mass vaccination has been partially helpful. However, mild and persistent viral infections are inducting huge losses to the productivity in birds. In this study, we evaluated the antiviral potentials of various regional plants against two common poultry viruses, the Infectious bronchitis (IB) and Infectious bursal disease (IBD). IB is an acute and highly infectious disease of chickens, which affects the respiratory tract. IBV can spread very rapidly to non-vaccinated birds. Infected chickens manifest symptoms like coughing, sneezing, tracheal rales, and nasal discharge. Respiratory irritation is also reported in young chickens. Reduced egg production accompanied with other symptoms in layers of the flock also result from such viral infection (Shahzad *et al.*, 2011). IBD virus belongs to genus *Avibirnavirus* and family *Birnaviridae*. This disease is also called as Gumboro in a local Cholistan language. It affects the *bursa of Fabricius* in birds. Broilers can be affected at any stage of their life, however, poultry birds 3-6 weeks of age are highly prone to this disease (Jackwood *et al.*, 2011). In 2008 numerous incidents of IBD were recorded with severe consequences. The commercial egg industries were more affected than broiler chickens (Khan *et al*, 2009). Despite the availability of massive vaccination campaigns, both these diseases are prevalent in different parts of the country. Our data suggest, that besides vaccination there is a possibility of using plant extracts having antiviral activity in poultry feeds for overcoming the threat from both of these viruses which are posing a threat to the poultry industry.

MATERIALS AND METHODS

Identification and collection of plants: Eleven fresh plants (*Achyranthes aspera*, *Haloxylon recurvum*, *Haloxylon salicornicum*, *Oxystelma esculentum*, *Ochthochloa compressa*, *Neurada procumbens*, *Panicum*

antidotale, *Salsola baryosma*, *Suaeda fruticosa*, *Sporobolus icolados* and *Solanum surattense*) were collected from the herbarium of Cholistan Institute of Desert Studies (CIDS), The Islamia University of Bahawalpur, Pakistan and near the Lal Sohanra region of Cholistan Desert of Punjab, Pakistan. The plants were collected and used as a whole in this study. The specimens were cleaned and dried under the shade, at room temperature (RT) for 10 days. Two poultry viruses including vaccinia strains of IBV named as Izovac IB H120 from IZO S.U.R.L 99/A-25124 Brasica Italy and IBD named as DS GumboroVac from Dae Sung Microbiological Laboratories, Seoul, South Korea were used in this study.

Plant Extracts Preparation: Upon complete drying, the plants were cut into small pieces, ground by pestle and mortar and finally ground to powdered form by using an electric grinder. These powders were kept in airtight containers at room temperature. Ten grams of powder from each plant was dissolved in 200 ml methanol. The solutions were kept in airtight containers for 96 hours, with constant shaking. These samples were subjected to a rotary evaporator (45-50°C). After evaporation, the concentrate was rinsed with a methanol and chloroform mixture and allowed to evaporate once again. Finally the precipitates were dissolved in 10 ml sterile water, filter sterilized and stored at -20°C in accordance with the protocol described previously (Joshi and Kaur, 2013).

Inoculation of poultry viruses in chicken embryonated eggs: Seven to eleven day old chicken embryonated eggs were taken from a local hatchery and used in the inoculation studies. The eggs were candled before inoculations. Both the viruses were inoculated in the chorioallantoic fluid of the eggs. The broader ends of the eggs were drilled with a sterile needle. After inoculation, the hole was sealed with molten wax and eggs were incubated at 37°C. The allantoic fluids were harvested 48 hours post inoculation (PI) and subjected to a HA test in case of IBV and an IHA test for IBDV. Serial passages of viruses were done to increase titer before subjecting them in antiviral assay.

In Ovo Antiviral Assay

Hemagglutination Test: Chicken blood was collected in freshly prepared Alsevier solution and centrifuged at 4000 rpm for 5 min. The supernatant was discarded and RBCs were washed with phosphate buffered saline (PBS) (pH 7.2). This step was repeated three times. To prepare 1% suspension, 10 µl of packed cells were dissolved in 1ml PBS (pH 7.2). After that, the cells were used to perform a standard HA test (Hirst, 1942).

Indirect Hemagglutination (IHA) Test: Human blood type "O" was used in the IHA test. For this purpose 3 ml of blood was drawn in a 4% sodium citrate solution and mixed gently. The sample was centrifuged at 4000 rpm for 5 min and supernatant was removed. The packed cells were dissolved in 10 ml of PBS (pH 7.4) and suspension was centrifuged again at 4000 rpm for 5 min. This procedure was repeated three times. After washing, the cells were sensitized by mixing with an equal volume of virus and two volumes of PBS (pH 7.4). The contents were mixed gently and incubated at 37°C for 45 min.

After incubation, the sample was centrifuged at 4000 rpm and supernatant was removed. The cells were washed with PBS (pH 7.4) once again and finally, diluted with PBS (pH 7.4) to make 1% suspension (Okwor *et al.*, 2012). Later, the standard HA test was performed by using these sensitized cells by following the previously described procedure (Hussain *et al.*, 2003).

Antiviral assay: The filter sterilized plant extracts were mixed with an equal volume of viral inoculums and injected in 7-11 day old chicken embryonated eggs as the method described by Rajbhandari *et al.* (2001). Filter sterilized H₂O was used in the case of a negative control and viruses without any plant extracts were used in the case of virus control. The eggs were harvested 48 hours post-infection and HA and IHA tests were performed.

All the protocols described in this study were approved from departmental biosafety committee of the Islamia University of Bahawalpur, Pakistan

RESULTS

Antiviral activity against IBV: Among the eleven plant extracts, selected for their antiviral effect, with the exception of *S. surattense* all plants were effective in controlling IBV virus in embryonated eggs, however, the antiviral activity varied from plant to plant. In this paper the antiviral effect is reported in terms of HA/IHA titers, higher

the HA/IHA titer value in the presence of plant extract, the lower the antiviral effect. The two plant extracts *O. compressa* and *S. icolados* methanolic extracts exhibited optimal antiviral activity (HA titer = 0) showing complete control over viral growth (Figure 1). The relative antiviral effect for other plants used in this study were: *A. aspera*, *H. recurvum* and *P. antidotale*, HA titer value 6; *H. salicornicum*, *N. procumbens*, and *S. baryosma*, HA titer value 8. The methanolic extract of *O. esculentum* relatively showed a lower antiviral effect manifesting an HA titer value of 32 followed by *S. fruticosa* with HA titer 64. Overall ten out of eleven plants were found effective against IBV varying in their antiviral potential (Fig. 1 and 3A).

Antiviral activity against IBDV: The antiviral potentials of eleven plants used in this study against IBDV was determined in term of indirect hemagglutination assay (IHA). Nine out of eleven plants were effective in controlling growth of IBDV (Fig. 2). The methanolic extracts of *A. aspera*, *H. recurvum*, *H. salicornicum*, *P. antidotale*, *S. baryosma*, *S. icolados* had kept the IHA titer to zero manifesting no viral growth. Similarly, *N. procumbens* had reduced viral growth with an IHA value of 2 followed by *S. surattense* manifesting a relatively lower control with IHA 4. The methanolic extract of the other plants used in this study like *S. surattense*, *O. esculentum* and *O. compressa* partially inhibited IBDV growth in the embryonated eggs model used in this study (Fig. 2 and 3B).

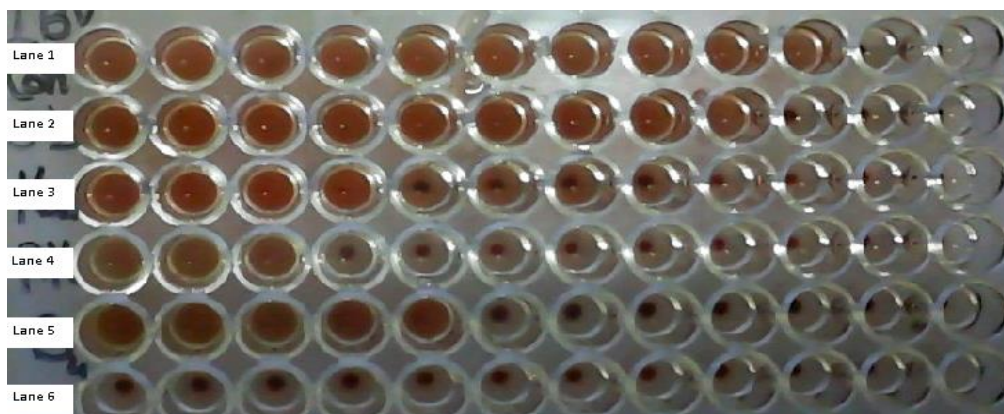


Fig 1: Representative antiviral activity of selected medicinal plants used in this study – methanolic extracts against IBV. Lane 1: HA titer of IBV without plant extract (virus control). Lane 2: HA titer of IBV after challenge with extract of *S. baryosma*. Lane 3: HA titer of IBV after challenge with extract of *S. surattense*. Lane 4: HA titer of IBV after challenge with extract of *A. aspera*. Lane 5: HA titer of IBV after challenge with extract of *O. esculentum*. Lane 6: PBS (negative control).

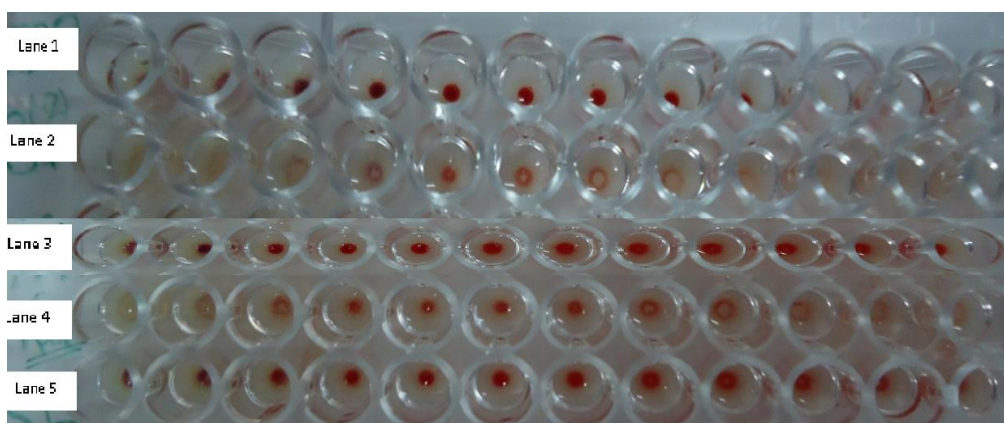


Fig 2: Representative antiviral activity of selected medicinal plants used in this study – methanolic extracts against IBDV. Lane 1; IHA titer of IBDV without extract (virus control), Lane 2: IHA titer of IBDV challenged with extract of *A. aspera*. Lane 3: IHA titer of IBDV challenged with extract of *O. esculentum*. Lane 4: IHA titer of IBDV challenged with extract of *S. surattense*. Lane 5: IHA titer of IBDV challenged with extract of *S. baryosma*.

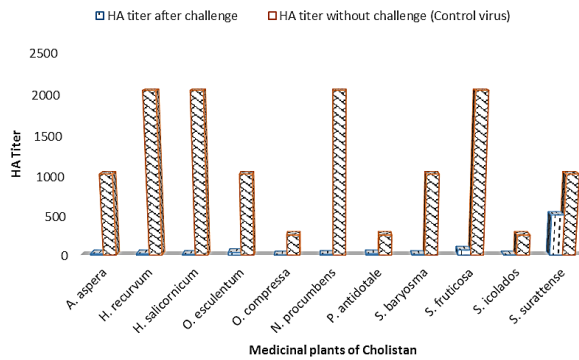


Fig 3A: Antiviral activity of different medicinal plants against IBV

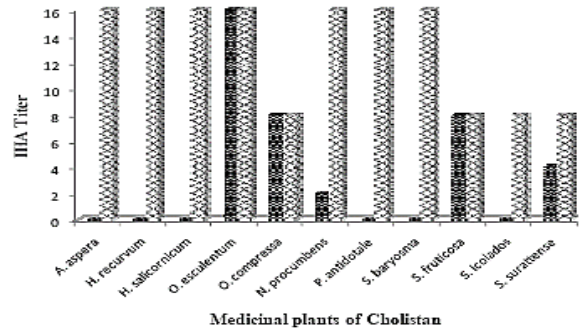


Fig 3B: Antiviral activity of different medicinal plants against IBDV

DISCUSSION

In the history of mankind, for thousands of years, traditional medicines including medical plants were the ultimate source for treating living organisms including humans suffering from infectious and non-infectious disease. According to WHO, still more than 80% of people around the world depend on traditional medicines obtained from herbs (Arunkumar and Muthuselvam, 2009). According to historical ethno-botanical data the plant extracts, infusions and powders of medicinal plants were used against infectious diseases for centuries and now it has been confirmed that a number of these diseases are of viral origin (Sumithira *et al.*, 2012). In the current study methanolic extracts of eleven Cholistan plants were prepared and tested against infectious bronchitis virus and infectious bursal disease virus the two viruses causing economic losses to the poultry industry all across Asia. For viral infectivity assays both viruses were recovered from live attenuated vaccines and propagated in embryonated eggs to check their viability. The plants used in this study have been used as either traditional medicines by the natives of this region and their properties as antibacterial, antifungal, anticancerous, antiperiodic, diuretic, purgative, laxative, antiasthmatic, hepato-protective, anti-allergic and other important medicinal properties have been reported. As far as antiviral potential(s) of these plants is concerned our study is a pioneering one. According to this study, selected Cholistan plants were very effective in controlling the growth of poultry viruses under *in vitro* conditions especially in their methanolic extract (crude form). On the basis of results, these plants were divided into four groups. 1) The plant extracts (crude extracts) that are highly effective against both viruses and those that kept the HA and IHA titer (a measure of viral replication) between 0 & 2) Plant extracts effective against both viruses, but that did

not control the HA and IHA titer significantly. 3) The plant extracts effective against only one virus. 4) Plant extracts least effective against both viruses. The plant *O. icolados* belongs to group 1 because it showed a significant inhibitory effect on both poultry viruses used in this study. Similarly, *S. baryosama*, *N. procumbens*, and *H. salicornicum* belong to group 2, as their methanolic extracts were partially effective in controlling viral growth. (Fig. 3A and 3B). *A. aspera*, *H. recurvum*, and *P. antidotale* were effective in controlling IBDV but less effective in controlling IBV, similarly, *O. esculentum*, *O. compressa* and *S. fruticosa* were effective in controlling IBV but ineffective in controlling growth of IBDV and as such classified in group 3. The plant *S. surattense* belong to group 4 because it was least effective in controlling the growth of either virus. Although, our study is preliminary in nature as the antiviral components of plants showing significant inhibitory effect on both these viruses still needs to be deciphered. To the best of our knowledge, this is the first report in scientific literature describing the antiviral potential of plants from the Cholistan desert, one of the living deserts in the world having significant flora and fauna. Furthermore, control of viral replication by some plants is quite intriguing and needs to be explored further. There is huge potential for translating traditional plant usage into modern drug discovery practices for humans, animals and domesticated birds like the poultry industry. It is timely to integrate all the available knowledge, especially relevant to viral infections, particularly in the wake of their zoonotic nature. There are several reports on plant based antiviral compounds against poultry viruses like Fucoidan from *C. Okamuraanus*- a desert plant, against new castle disease virus (NDV) (Gonzalez *et al.*, 2012). Premnathan *et al.* (1992) data reveal antiviral activity of different seaweeds, seagrasses and mangroves in the southeast coastal region of India against NDV, vaccinia and hepatitis B viruses. Similarly, (Usha *et al.*, 2012) aqueous, ethanolic and methanolic extracts of lathakaranja (*C. crista* L.) have been tested and these extracts possess antiviral effects against NDV (Chollom *et al.*, 2012). Not only plants but the fruit pulp and leaf extracts of *Momordica balsamina* exhibited an antiviral effect against NDV. The ethanolic extracts of different plants including *Glycyrrhiza glabra*, *Moringa oleifera*, *Phyllanthus emblicus* and *Eugenia jambolana* against IBDV (Ahmed *et al.*, 2014). Along similar lines, aqueous and alcoholic extracts of *Moringa oleifera*, *Holarrhena antidysenterica*, *Synzium aromaticum*, *Allium sativum*, *Piper nigrum* and *Azadirachta indica* were effective in controlling IBDV growth. (Meenakshi *et al.*, 2009) The anti-IBDV activity from the root of *Withania somnifera* in its hydro-alcoholic extracts has also been reported (Pant *et al.*, 2012). There is plenty of literature on the antiviral potential of plants, however, what is needed is translating data into useful products. Differential antiviral effect as observed in our study has also been reported by other researchers. For example crude ethanolic extracts of *Rhodiarosea* roots, *Nigella sativa* seeds, and *Sambucus nigra* fruit was effective against IBV in varied order (Chen *et al.*, 2014). Similarly, aqueous extract of *Rhododendron ferrugineum* was effective against HSV-I but least effective against adenovirus (Gescher *et al.*, 2011). The extract had provided an anti-adhesive effect on the virus and did not allow the virus to enter the cells. The proanthocyanidins

and flavonoids like polyphenols were found active in providing anti-adhesive effects and these effects can be varied by change in amino acid sequence and hydrophobicity of antigenic proteins on the viruses (Frazier *et al.*, 2010). Anti-adhesive activity from plant extracts is a new concept in the field of antiviral research and it needs to be further studied to understand the extent to which different viruses are controlled by anti-adhesives (polyphenols). Some studies against HSV, AIV and enteroviruses provide the basis for further research on plant based anti-adhesive compounds (Ho *et al.*, 2009). Another reason to use plant products is the mode of action that these compounds exert, the plant based products, work in a synergistic way, thereby providing less time for microbes to mutate and to develop resistance against drugs. Despite the availability of good vaccines, the demand for antiviral compounds is rising. Moreover, extensive use of vaccines could be potential sources of genetic variation in microbes, therefore, screening for new and more effective antimicrobial compounds from plants or any other natural source should be the focus.

Conclusions: Plants growing in Cholistan desert of Pakistan are rich source of antiviral agents. The extracts of these plants can be used in crude form to combat viral infections in poultry and livestock industry in case of outbreak. Furthermore, deserts with similar habitats are also found in Middle East including United Arab Emirates and several other parts of the world. A highly integrated and collaborative much needed antiviral discovery program from natural resources from these areas is timely to combat emerging and existing viral infections.

Author's contribution: MIS and MK designed this study analyzed and interpreted the data. NN and SSZ collected and verified plants. AA, SP, HA and ZK performed lab experiments. All authors read and revised the manuscript.

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