

## REPORT

# Some ethanobotanically important plants from Cholistan area for anti avian influenza virus (AIV) H9N2 screening

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**Abstract:** Avian influenza or bird flu is a common problem of domestic and wild birds. Some of its strains are able to cross the species barrier and cause infection in various members of class Mammalia. In view of relatively lesser efficacy of vaccines, antiviral therapies remain the only choice for the sustenance of mammals acquiring this highly devastating infection. This study is based on the evaluation of antiviral potential of methanol extracts of eleven selected Cholistan plants. The methanol extracts were prepared by using dried plants material followed by concentrating in a rotary evaporator and finally air dried before dissolving in nanopure water. The suspension was filter sterilized and subjected to *in ovo* antiviral assays. The allantoic fluids were harvested and haemagglutinin (HA) titers were determined. Among the eleven plants evaluated all methanol extracts were found effective against AIV H9N2 except *S. baryosma* extract. The medicinal plants *O. compressa*, *N. procumbens*, and *S. surattense* were found to be more effective than others and they retained HA titers at 0 after challenge. The next in order were extracts of *O. esculentum*, *H. salicornicum* and *S. fruticosa* which kept HA titers at 4, 8 and 16 respectively. The extracts of *H. recurvum*, *P. antidotale*, *S. icolados* and *A. aspera* were found less effective than above mentioned plant extracts and they kept the HA titers at 32, 64, 128 and 256 respectively. These results led us to conclude that the medicinal plants of Cholistan region are a rich source of antiviral agent(s) against AIV H9N2 and could be a source of cost effective alternate therapeutics.

**Keywords:** Cholistan, medicinal plants, antiviral, AIV H9N2.

## INTRODUCTION

Avian Influenza viruses (AIV), has segmented negative strand RNA genome belonging to *Orthomyxoviridae* family. Birds and mammals are the primary host of this virus (Swayne and King, 2003). This virus has three subtypes i.e. A, B and C, each classified on the basis of nucleoprotein and matrix proteins. Variation in surface glycoproteins, hemagglutinin (H) and neuraminidase (N) results in further division of subtype. A total of 16 H and 9 N proteins are identified in aquatic wild birds and other animals so far. Wild waterfowl is a natural reservoir for the virus and transmits infection to poultry (Swayne and King, 2003). Only a few subtypes are highly pathogenic originating from persistently infecting low pathogenic strains in birds. A number of AIV outbreaks have been reported in Pakistan. The highly pathogenic strain H7N3 was reported during 1995-2003, causing 3.2 million

deaths in birds (Naeem *et al.*, 1999). Less pathogenic strain H9N2 still prevails in avian stocks of Pakistan. Numerous outbreaks of less pathogenic H9N2 were recorded in Pakistan (Naeem *et al.*, 1999).

Traditional utilization of plants as a source of medicine is a common practice in developing countries including Pakistan, Africa, China, Sri Lanka, India and Thailand (Hoareau & Da Silva, 1999). According to Natural Health Product Regulations of Canada, 2004, the trend to use medicinal plants is also gradually increasing in developed countries like North America and Europe. These countries are now accepting wide range plant based health products (Siow *et al.*, 2005). According to a study, only 5% of plant species have been screened as a potential source of medicines while the rest still need to be analyzed (Mukherjee, 2004). Herbal preparations are also in use since time immemorial for treating a variety of chronic ailments. Recently Vlietinck *et al.* (1998); Matthe *et al.* (1999) and Mlinaric *et al.* (2000) have studied plant based

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anti-HIV compounds from selected medicinal plants and accordingly antiviral activity against HIV is available through different medicinal plants worldwide. Similar studies done by Bedoya *et al.* (2001) on plants from Iberian Peninsula and screened various medicinal plants of this region for their anti HIV activity. Zhu and Ren (1995) reported antiviral activity in extracts of *Geranium carolinianum* L. and its aqueous extract is good against HBV and helps in improving the clinical data of patients. Plants are natural reservoir of compounds like tannins, flavonoids, alkaloids, polyphenols, antiadhesives, proanthocyanidins etc. some of these compounds are successfully tested against different viruses like HIV, HSV, HBV and NDV (Namba *et al.*, 1989; Khan and Ather, 2005 and McLaughlin and Paw, 2008; Mukhtar *et al.*, 2008; Shahzad *et al.*, 2019). Plant based polyphenols were found effective against HSV (Shahat *et al.*, 2002).

Medicinal plants have already been used in treating viral infections of both humans and animals since long ago (Hudson, 1990). These plants (as whole plant, extracts or powders) are in use to treat different bacterial, fungal and viral infections ever since the recorded history of mankind. Demand for new antimicrobial agents is on continuous rise especially antiviral agents. This demand is due to shortage of antiviral agents in market and emergence of resistance against the existing drugs (Vijanyan *et al.*, 2004). Higher plants are a rich source of antiviral agents and many of these compounds can serve as antiviral prototypes (Cowan, 1999). A new compound Nordihydroguaiaretic acid (NDGA), was isolated from leaves of *Larrea divaricata* and it was found effective against HIV, Junin virus and HSV type I and II (Konigheim *et al.*, 2005). Similarly, number of other novel antiviral compounds like flavonoids, lignans, coumarins, furyl compounds, peptrides/polypeptides and proteins, polyphenolics, sulfides, saponins and terpenoids, have already been reported from plants. Some plants have shown antiviral activity in their aqueous extracts and are used as herbal teas while some have shown antiviral activity in oils extracted from their different parts (Cowan, 1999). Keeping in view the natural resourcefulness of plants and comparatively less data available from Cholistan plants, this study was designed to evaluate Cholistan plants antiviral potential against AIV H9N2. This strategy provides easy and cost effective solutions to address viral outbreaks in poultry and livestock industry as well as to generate cost effective alternate medicines from local plants.

## **MATERIALS AND METHODS**

### ***Specimen collection***

Eleven fresh plants named *Suaeda fruticosa*, *Solanum surattense*, *Achyranthus aspera*, *Ochthochloa compressa*, *Panicum antidotale*, *Haloxylon recurvum*, *Haloxylon salicornicum*, *Oxystelma esculentum*, *Sporobolus icolados*

*Neurada procumbens* *Salsola baryosma* were collected from Cholistan desert area surrounding the Islamia University of Bahawalpur, Pakistan as a whole plant. The plants identification and authentication was confirmed by Mr. Ghualm Sarwar, lecturer Department of Life Sciences, The Islamia University of Bahawalpur.

### ***AIV H9N2***

AIV H9N2 was re-activated from live attenuated vaccine named as Gallimune Flu H9 M. E from Merial Laboratories Italy. The vaccine was Alum precipitated and Alum was removed by centrifugation and supernatant was used for chick embryonated egg inoculations. The virus was activated after serial passages in 7 to 11 days old chick embryonated eggs.

### ***Preparation of methanol extracts***

The freshly collected plants were rinsed with water and completely dried at room temperature (RT) for 12 to 15 days. Plant parts were cut into small pieces and ground to powder form using an electric grinder. The dried powdered plants were kept in airtight containers at RT till further used. To obtain methanol extract of each plant Joshi and Kaur, (2013) method was used. Ten grams of powdered plant was dissolved in 200 mL of 100% methanol and solution was kept at shaker for 96 h. Rotary evaporator (45-50°C) was used to evaporate methanol, and precipitates were rinsed with methanol and chloroform turn by turn and allowed them to re-evaporate. Finally, the precipitates were suspended in distilled water, shaken well and filtered. All extracts were filter sterilized by passing through syringe filters and stored at -20°C.

### ***Egg inoculations***

Seven to eleven days old specific pathogen free (SPF) chicken embryonated eggs were used for virus propagation. The viability of embryos was checked by candling before inoculations. All the egg inoculations were done in Biosafety Cabinet type II. The broader ends of eggs were swabbed with 70% alcohol and tiny hole made by using sterile common pin. Inoculation was done immediately and hole was sealed with molten wax. Eggs were incubated at 37°C. These eggs were harvested, 48 h post inoculations (PI) and HA test was performed. Serial passages of virus were done to fully activate virus before taking it into antiviral assays.

### ***Hemagglutination Test (HA)***

HA test was performed, as the method described by Hirst, 1942.

### ***In ovo antiviral assay***

Each plant extract was mixed with equal volume of viral inoculum and inoculated in embryonated eggs as the method described by Rajbhandari *et al.*, (2001). Autoclave distilled water was used in case of negative control and AIV H9N2 was used as virus control. Eggs were harvested 48 h PI and HA titers were noted (table 1).

## STATISTICAL ANALYSIS

The mean and standard deviations were calculated by software Calculator.net.

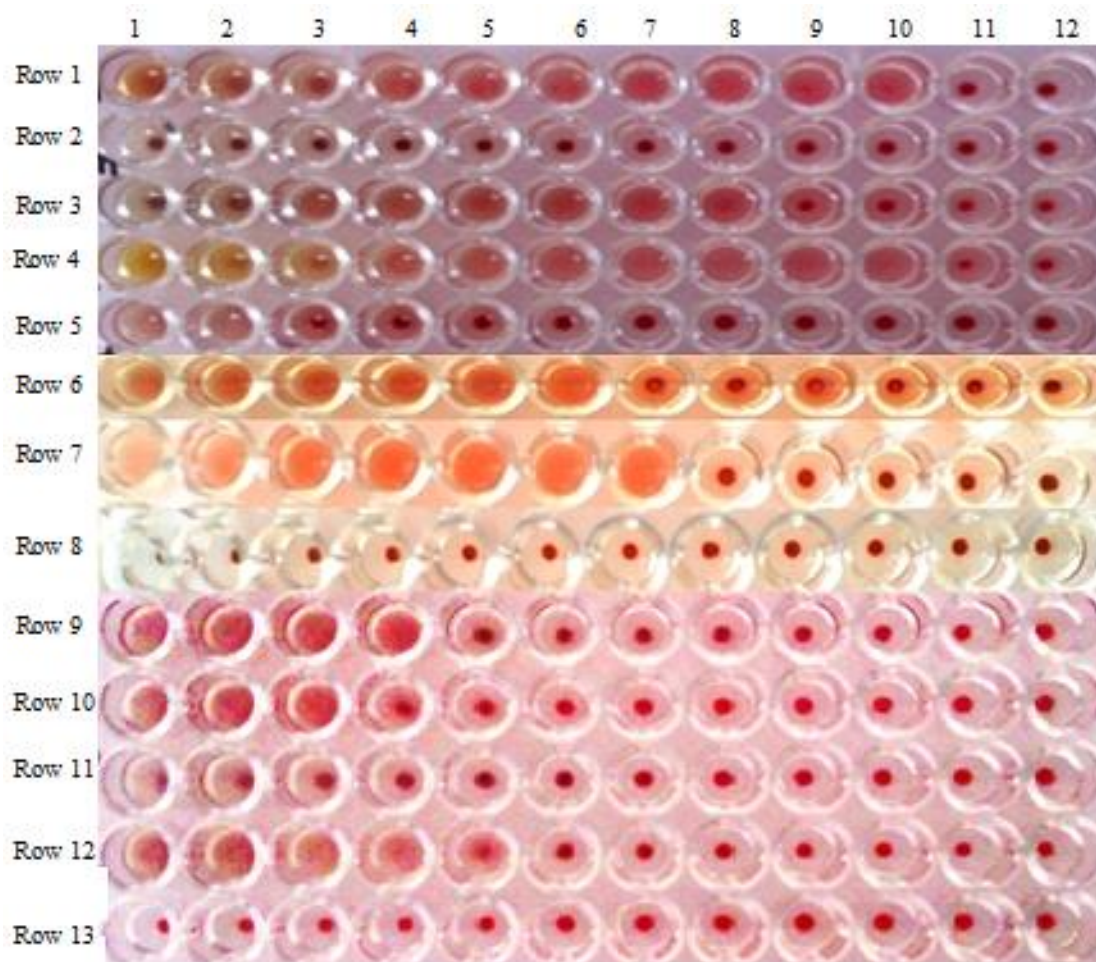
## RESULTS

### Cultivation of AIV-H9N2 virus

According to results of cultivation experiment of AIV-H9N2, HA titer was 0 after 1<sup>st</sup> passage. The virus started producing HA titer after 5<sup>th</sup> passages and after 8<sup>th</sup> passage HA titer was 128. After 10<sup>th</sup> passage, HA titer was 1028 (fig. 1, Row 1).

### Antiviral assay from different Cholistani plants against AIV-H9N2

According to results, the methanol extracts of *S. surattense*, *O. compressa* and *N. procumbens* were best among all as far as the antiviral effect is concerned. The HA titers were 0 in all cases (fig. 1A, Row 2, 8 & 11). Zero HA titer means, these extracts did not allowed the virus to grow at all. The methanol extracts of *O. esculentum* and *H. salicornicum* were also very protective in controlling AIV infection in embryonated eggs. The HA titer from these extracts were 4 and 8 (fig. 1, Row 5 & 10). Next in order of antiviral potential, the methanol extracts *S. fruticosa*, *H. recurvum*, *P. antidotale* and *S. icolados*. These plant extracts had reduced the viral load significantly and their respective HA titers were 16, 32,



**Fig. 1:** Antiviral assay from methanol extracts of different Cholistani plants. Row 1: HA titer of H9N2 virus. Row 2: HA titer of H9N2 after treatment with methanol extract of *S. surattense*. Row 3: HA titer of H9N2 after treatment with methanol extract of *A. aspera*. Row 4: HA titer of H9N2 after treatment with methanol extract of *S. baryosma*. Row 5: HA titer of H9N2 after treatment with methanol extract of *O. esculentum*. Row 6: HA titer of H9N2 after treatment with methanol extract of *P. antidotale*. Row 7: HA titer of H9N2 after treatment with methanol extract of *S. icolados*. Row 8: HA titer of H9N2 after treatment with methanol extract of *O. compressa*. Row 9: HA titer of H9N2 after treatment with methanol extract of *S. fruticosa*. Row 10: HA titer of H9N2 after treatment with methanol extract of *H. salicornicum*. Row 11: HA titer of H9N2 after treatment with methanol extract of *N. procumbens*. Row 12: HA titer of H9N2 after treatment with methanol extract of *H. recurvum*. Row 13: PBS (negative control).

**Table 1:** Antiviral activity of selected Cholistani plants through methanol extracts with mean and standard deviations

Plant used	HA titer after challenge			Mean	S. dev	Virus control
<i>S. surattense</i>	0	0	2	0.66666667	1.15470054	1024
<i>A. aspera</i>	256	256	256	256	0	2048
<i>S. bryosoma</i>	1024	512	1024	853.3333333	295.6033338	1024
<i>O. esculentum</i>	4	4	8	5.333333333	2.30940108	1024
<i>P. antidotale</i>	64	64	128	85.33333333	36.9504172	2048
<i>S. icolados</i>	128	128	128	128	0	1024
<i>O. compressa</i>	0	0	2	0.66666667	1.15470054	1024
<i>S. fruticosa</i>	16	8	16	13.33333333	4.61880215	1024
<i>H. salicornicum</i>	8	8	8	8	0	1024
<i>N. procumbens</i>	0	0	0	0	0	1024
<i>H. recurvum</i>	32	32	64	42.6666667	18.4752086	2048

64 and 128 (fig. 1, Row 5 & 10). Seemingly, these potentially antiviral phytomedicine (crude extracts) were moderately effective in controlling growth of AIV H9N2 strain *in ovo*. The last plant which was effective in controlling growth of AIV H9N2 was *A. aspera*. Its crude extract has reduced the HA titer of virus till 8<sup>th</sup> well and its HA titer was 256 as compared to virus control (fig. 1, Row 3). The overall results are depicted in table 1.

## DISCUSSION

Pakistan ranks 7<sup>th</sup> among poultry producing countries and within the country it is considered as the second biggest. One of the challenges faced by poultry industry in Pakistan is concurrent infections of viruses like AIV (Anjum *et al.*, 1993). Sudden outbreaks of AIV and Infectious Bronchitis Virus (IBV) and persistent infections of infectious bursal disease virus (IBDV) and New Castle disease virus (NDV) have devastating effects on poultry industry. Very high morbidity and mortality rates have been reported in areas where disease is uncontrolled (Lukert *et al.* 1997). In case of sudden outbreak antiviral drug treatment therapy is the only choice. The drugs can be used in pure or crude form. Cholistan plants were well characterized for their medicinal importance and their activities like antifungal, antibacterial, diuretic, antiperiodic, anticancerous, hepatoprotective, antiasthmatic, laxative, purgative, anti-allergic but their antiviral potential was never explored. According to this study 10 out of 11 methanol extracts of Cholistan plants were found to inhibit replication of AIV H9N2 strain. The methanol extracts usually consist of small polar substances like alkaloids, flavonoids and terpenoids in suspension. All the extracts were used in equal concentration. The best antiviral activities were exhibited by extracts of *O. compressa*, *N. procumbens*, and *S. surattense*. All of them were equally effective in controlling AIV H9N2 (0 HA titer). The result indicates that these Cholistan plants share some common metabolite inside to control flu virus. Almost similar results were obtained from crude extracts of *O.*

*esculentum*, *H. salicornicum* and *S. fruticosa*. The HA titers 4, 8 & 16 respectively have clearly highlighted their potential against AIV H9N2. None of them were able to keep HA titer at 0, which means the current dose (100 µg) was not 100% effective in controlling growth of AIV. These plants have lesser quantity of antiviral agents or potency of their compounds was low could be the possible reason(s). The plants *H. recurvum*, *P. antidotale*, *S. icolados* and *A. aspera* with HA titers 32, 64, 128 and 256 respectively were next in order. The extracts of these plants were positive but their potency was further low. The exact activity of crude extracts has always remained questionable. Several studies have shown that increase in purity level of molecules, increase the biological activities of these moieties. The least effective plant in this list of medicinal plants was *S. bryosoma* and its HA titer was 1024. The cytotoxicity of methanol extracts was checked by monitoring the viability of embryos after each trial. None of the extract was found toxic for chick embryos. Histopathology of embryonic liver, lungs and kidneys were done and no gross changes on these organs were found. The results of this study are much similar to the results published by other researchers. Aslam *et al.* (2016) has reported anti-IBV and IBDV activities from Cholistan plants. Mothana *et al.* (2006) data showed antiviral activity from methanol and hot-aqueous extracts of nine medicinal plants of soqotra island of Yemen and they have tested these extracts against Influenza type A and herpes simplex type 1 viruses. In another study, Park, (2003) studied the antiviral activities from methanol extracts of four Korean medicinal plants against AIV. Similar results were reported against other poultry, livestock and human viruses. Droebner *et al.* (2007) confirmed the antiviral effects of plant based proanthocyanidins against different strains of AIV. Gonzalez *et al.* (2012) isolated and tested a plant based compound Fucoidan from *C. Okamura* against NDV. Similarly, methanolic extracts of mangroves, sea grasses and seaweeds of coastal region of South East India were tested successfully against NDV, vaccinia and HBV (Premnathan *et al.* 1992). Similarly, Usha and Sharma,

(2012) reported anti NDV activity from aqueous, ethanol and methanol extracts of *C. crista* L. (lathakaranja). Fruit pulp and leaf extracts of *Momordica balsamina* was tested against NDV by Chollom *et al.* (2012). Ahmed *et al.* (2014) studied anti IBDV activity from ethanol extracts of *G. glabra*, *M. oleifera*, *P. emblicus* and *E. jambo lana*. Similar results were reported by Meenakshi *et al.* (2009) and they used aqueous and alcohol extracts of *M. oleifera*, *P. nigrum*, *H. aantidysenterica*, *A. indica*, *S. aromaticum*, and *A. sativum* against IBDV through cell line based assay. According to Pant *et al.* (2012) hydro-alcoholic extract of *Withania somnifera* roots was full of antiviral compounds against IBDV. Similarly, the ethanolic extracts of *Rhodiola rosea* roots, *Sambucus nigra* fruit and *Nigella sativa* seeds were found active against IBV (Chen *et al.*, 2014). Researchers have suggested different mechanisms of antiviral compounds isolated from different medicinal plants; some has reported blocking effect or loss in attachment of the virus to host cell (Ho *et al.*, 2009) and others have reported inhibitory effect on essential enzymes (Rossignol, 2014). According to some researchers these antiviral compounds are peptides, polypeptide or protein in nature.

## CONCLUSIONS

This study highlights the medicinal importance of Cholistani plants growing the Cholistan Desert located in Southern Pakistan. The data strongly suggest that plant evaluated in this study are a rich source of antiviral compounds. The crude extracts of plants growing in this region can be used to combat viral infections in poultry and livestock industry. Furthermore, an organized farming of these medicinal plants is a dire need to promote the indigenous production of antiviral products for saving poultry industry within the country. Furthermore, farming of these plants subsequent to their authentication as potent antivirals can earn foreign exchange thus improving the economy of this region.

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