Antiviral, embryo toxic and cytotoxic activities of *Astragalus membranaceus* root extracts

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Abstract: Antiviral activity of *Astragalus membranaceus* aqueous and methanol root extracts was determined against Avian influenza H_9 virus. Toxicity profile of extracts was evaluated using chicken embryos and BHK-21 cell line. Different concentrations (400, 200, 100, 50, 25. 12.5, 6.25 and 3.12µg/mL) of both aqueous and methanol extracts were mixed with standard virus inoculum (4HAunits) and incubated for 30minutes at 37^{o}C prior to inject the chicken embryos. Chorioallantoic fluid harvested 72 hours post inoculation and evaluated for virus growth using hemagglutination assay. Same concentrations of both extracts without virus were injected in chicken embryos to evaluate embryo toxic activity as well. The cytotoxic activity of aqueous and methanol extracts was determined by MTT colorimetric assay using BHK-21 cells. Three concentrations (400, 200 and 100µg/mL) of aqueous and five concentrations (400, 200, 100, 50 and 25µg/mL) of methanol extract showed antiviral activity. None of the tested concentrations of aqueous and methanol *A. membranaceus* root extracts caused chicken embryo mortality. Cell survival percentage of aqueous extract was higher than 50 at all of the tested concentrations except 400µg/mL. Two concentrations (400 and 200µg/mL) of methanol extract showed cytotoxicity. It was concluded that aqueous and methanol roots extracts of *A. membranaceus* have antiviral activity and concentrations which were safe may be used for treatment of Avian influenza H_9 virus infections.

Keywords: *Astragalus membranaceus*, antiviral, embryo toxic, cytotoxicity, avian influenza H_9 virus.

INTRODUCTION

Plants are being used for many decades in healthcare for treatment of different microbial infections. Approximately, 80percent of the world's population relies mainly on plant based system for their primary healthcare in developed countries (Cragg and Newman, 2005). A large number of drugs have been developed from plants. Major constituents of plants having antimicrobial activity include essential oils, flavonoids, glycosides, etc. Efficacy of phytochemicals against viruses, fungi and bacteria is well established (Sengul *et al*., 2009; Hemaiswarya *et al*. 2008). Phytochemicals of *Astragalus membranaceus* have immune modulatory effects. It has been used for the treatment of fatigue, common cold, anorexia and diarrhea. Polysaccharide ingredient of *A. membranaceus* roots has therapeutic effects and is widely used as anti-diabetic, immune modulator, hepatoprotective, antiviral, antioxidant and anti-inflammatory (Jin *et al*., 2014). Cyclo lanostane-type saponi components (polysaccharides astragalans I & II and glycans AMem-P & AMon-S) of the plant play role in regeneration of hepatocytes and lymphocytes (Kondeva-Burdina *et al*., 2015; Yang *et al*., 2005).

Avian influenza (AI) virus belongs to Orthomyxoviridae, RNA virus and its proteins can be changed due to mutation. Subtypes H_7 and H_9 of AI virus are highly virulent in chicken and turkeys (Shaukat *et al*., 2011). Most common strategic option to treat virus infections is the use of synthetic chemicals. However, as an alternate option for treatment of viral diseases biomolecules extracted from medicinal plants is in use as well (Shin *et al*., 2010). Herbal drugs inhibit the AI virus entry into host cell. In the present study, activity of both aqueous and methanol *A. membranaceus* extracts against AI H_9 virus was evaluated. Toxicity profile of extracts was determined using chicken embryos and baby hamster kidney (BHK-21) cell line.

MATERIALS AND METHODS

Plant extracts

Astragalus membranaceus dried roots procured from local market of Lahore and authenticated from Department of Botany, Government College University, Lahore with voucher No. 3494. These roots were ground to fine powder. Phytochemicals were extracted using water and methanol as solvents in soxhlet apparatus (CG-1368). Extracts were filtered through muslin cloth and centrifuged at 200 rpm for 20 minutes (Singh *et al*., 2015; Kumar *et al*., 2013). Supernatants were collected and dried using Rotary evaporator (Stuart RE-300). Both methanol and aqueous root extracts of *A. membranaceus* were dried to semisolid mass at 40°C temperature using rotary evaporator (Bimaki *et al*., 2011).

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Avian influenza H_9 virus

Known Avian influenza virus H_9 ($AIVH_9$) procured from Department of Microbiology, University of Veterinary and Animal sciences, Lahore was used to assess antiviral activity of plant extracts. The virus was confirmed by Hemagglutination inhibition test following the protocol described by Pedersen (2008) using known antibodies. Standard concentration (4HA) of virus was prepared in phosphate buffer saline (PBS) on the basis of hemagglutinating (HA) activity (4HA) of AI virus for washed chicken erythrocytes (1%) using micro titration plate as described by Killian (2008). Two fold serial dilutions of AI virus were prepared, erythrocyte suspension mixed in each, kept for 20minutes at 37^{o}C and the highest dilution of virus which showed hem agglutination was considered one HA unit.

Antiviral activity

Eight different concentrations of *A. membranaceus* aqueous and methanol extracts (400, 200, 100, 50, 25, 12.5, 6.25 and 3.125µg/mL) were mixed with standard concentration of the virus and incubated for 30 minutes at 37^{o}C for antiviral activity (Song *et al*., 2015). Each of the virus and extract mixture was injected in 9-day old chicken embryos (n=03) procured from Hi-Tech laboratories, Lahore, using allantoic cavity route under sterilized conditions. Three embryos were injected with PBS only (the negative control) and three injected with the virus alone (positive control). Inoculated eggs were incubated at 37^{o}C with 60% humidity in incubator. Eggs were examined daily using egg candler to observe embryo mortality. Chorioallantoic fluid was collected in sterilized test tubes to check the presence of virus using spot hemagglutination and micro dilution method for growth of the virus.

Embryo toxicity

To determine embryo toxicity same concentrations of both the extracts without virus were inoculated in chicken embryos following Hong *et al*. (2015). Embryo mortality caused by extracts was recorded for three days.

Cytotoxicity

Concentrations of both extracts used to check cytotoxic activity included: 400, 200, 50, 25, 12.5, 6.25 and 3.125µg/mL following Ge *et al*. (2015). Micro-titration plate (flat bottom) was used to culture baby hamster kidney (BHK-21) cells in the form of monolayer. A volume of 100µL of each extract (concentration) was poured in triplicate over BHK-21 monolayer and incubated for 96hours at 37°C. MTT (3-4, 5dimethylthiazole-2 yl)-2, 5 diphenyl tetrazolium bromide) 0.5 percent solution (100µL) was poured in each well and incubated at 37°C for 4hours. MTT dye was replaced with 10% DMSO (100µL) and incubated for two hours at 37°C. OD_{570} (of plate) was measured by multi well ELISA reader along with positive and negative control wells (Harne *et al*., 2012). Safety of extracts was calculated on the basis of cell survival using formula**:** Mean OD of test- Mean OD of -ve control/ Mean OD of +ve control x 100.

STATISTICAL ANALYSIS

Data of antiviral activity and O.D. values of cytotoxicity were analyzed using One way ANNOVA followed by Duncan's Multiple range posthoc test by Statistic Package for Social Sciences, version16.0.

RESULTS

Antiviral potential of *Astragalus membranaceus* against Avian Influenza (AI) virus was evaluated and out of eight tested aqueous extract concentrations only three (400, 200 and 100µg/mL) inactivated the virus. These concentrations showed higher antiviral activity and hence no virus growth was observed. Five of the tested concentrations (400, 200, 100, 50 and 25µg/mL) of methanol extract were found to be antiviral while other three the five did not stop the viral replication (table 1). Growth of the virus in concentrations of aqueous and methanol extracts detected by spot hemagglutination test in harvested chorioallantoic fluid as indicated by agglutination of washed erythrocytes is presented in fig. 1.

Embryo toxicity of both the extracts was determined by inoculating the selected concentrations in chicken embryos and none of the tested was found toxic. All of the tested concentrations proved safe and no embryo mortality was observed at any concentration.

The cytotoxic activity of both extracts was evaluated on baby hamster kidney (BHK-21) cells using MTT (3-4, 5dimethylthiazole-2 yl)-2, 5 diphenyl tetrazolium bromide) colorimetric assay. Cell survival percentages were calculated and concentrations in which survival were higher than 50percent was declared safe. In aqueous concentration of 400µg/mL cell survival percentage was lower than 50 whereas all other tested concentrations were found safe (table 2). Two concentrations (400 and 200µg/mL) of methanol extract showed cytotoxicity as cell survival percentage was lower than 50 and all other concentrations were found safe (table 2). It was concluded that roots extracts of *Astragalus membranaceus* had antiviral activity and safe concentrations could be used for treatment of avian influenza caused by AIH_9 virus.

DISCUSSION

Antiviral activity of different medicinal plants has been checked against Human immunodeficiency virus, Herpes simplex virus, Newcastle disease virus and Hepatitis B virus. Activity of *A. membranaceus* aqueous and

Table 1: Antiviral activity of *Astragalus membranaceus* aqueous and methanol root extracts against avian influenza virus (H_9) using chicken embryos

S. No.	Concentrations (µg/mL)	Replicates	Aqueous extract			Methanol extract		
			HA titer	Log 2 values	Mean±S.D	HA titer	Log 2 values	Mean±S.D
1	400	1	00	00	00.00±0.00[a]	00	00	00.00±0.00[a]
		2	00	00		00	00	
		3	00	00		00	00	
2	200	1	00	00	00.00±0.00[a]	00	00	00.00±0.00[a]
		2	00	00		00	00	
		3	00	00		00	00	
3	100	1	00	00	00.00±0.00[a]	00	00	00.00±0.00[a]
		2	00	00		00	00	
		3	00	00		00	00	
4	50	1	64	06	05.70±0.57[b]	00	00	00.00±0.00[a]
		2	64	06		00	00	
		3	32	05		00	00	
5	25	1	256	08	07.70±0.57[c]	00	00	00.00±0.00[a]
		2	256	08		00	00	
		3	128	07		00	00	
6	12.5	1	256	08	07.70±0.57[c]	32	05	05.70±0.57[b]
		2	256	08		64	06	
		3	128	07		64	06	
7	6.25	1	1024	10	09.70±0.57[d]	256	08	08.70±0.57[c]
		2	1024	10		512	09	
		3	512	09		512	09	
8	3.125	1	1024	10	09.70±0.57[d]	512	09	09.70±0.57[d]
		2	1024	10		1024	10	
		3	512	09		1024	10	
9	Only virus	1	1024	10	10.00±0.00[d]	1024	10	10.00±0.00[d]
		2	1024	10		1024	10	
		3	1024	10		1024	10	
10	Only PBS	1	00	00	00.00±0.00[a]	00	00	00.00±0.00[a]
		2	00	00		00	00	
		3	00	00		00	00	

Means within columns carrying same superscripts differ non-significantly whereas with different superscripts differ significantly ($P< 0.05$)

methanol extracts was observed against Avian influenza virus (H_9) during this study. Different concentrations of both extracts which showed antiviral activity were comparable to those reported by Shuang-Suo *et al*. (2006) for Hepatitis B virus (2.19g/L). Juan *et al*. (2007) checked activity of *Astragaloside* against extracellular Hepatitis B virus and found higher in comparison to lamivudine. Higher inhibition potential of plant was observed at lower order dose. Similarly higher survival rate of mice by *A. membranaceus* was reported by Feng-ving *et al*. (2005) with lower anomalies of cardiovascular system. It was concluded that *A. membranaceus* has antiviral activity and can be used in viral myocarditis. In view of present findings both plant extracts showed activity against AI virus in which mutational rate is much higher, hence extracts could be a better choice for the treatment of virus infections in poultry birds.

Extracts of *A. membranaceus* were found non-toxic for chicken embryos at different concentrations as no mortality was observed. Results of present study are in line with the findings of Lixia *et al*. (2006) whereby *Angelica sinensis* and *A. membranaceus* were safe in chick embryo chorioallantoic membrane model. Shabbir *et al*. (2008) observed immune modulatory effect of *A. membrananceus* in poultry birds. Immune suppression caused by live attenuated infectious bursal disease virus vaccine in broilers was masked by *A. membranaceus* plant extract. Immune efficiency and growth rate observed were markedly higher in treated group. In agreement results had been reported by Juan el al. (2007) on the basis of Hepatitis B virus replication inhibition using cell culture model by *Astragaloside.* Inhibition of virus observed was dose dependent without any toxicity of plant extract as determined by MTT assay. Antiviral

Table 2: Cytotoxic activity of *Astragalus membranaceus* aqueous and methanol root extracts using BHK-21 cell line by MTT assay

S. No.	Concentrations (µg/mL)	Aqueous extract		Methanol extract	
		Mean O.D.±S.D	CSP	Mean O.D.±S.D	CSP
1	400	1.416± 0.722	38	0.845± 0.076	9
2	200	1.704± 0.541	51	1.039± 0.058	19
3	100	1.785± 0.539	55	1.704± 0.541	51
4	50	1.798± 0.490	56	1.785± 0.539	55
5	25	1.837± 0.534	60	1.798± 0.490	56
6	12.5	1.853± 0.561	64	1.837± 0.534	60
7	6.25	1.973± 0.174	70	1.853± 0.561	64
8	3.125	2.357± 0.539	85	1.973± 0.174	70

Means within columns carrying same superscripts differ non-significantly whereas with different superscripts differ significantly ($P< 0.05$) CSP: Cell survival percentage

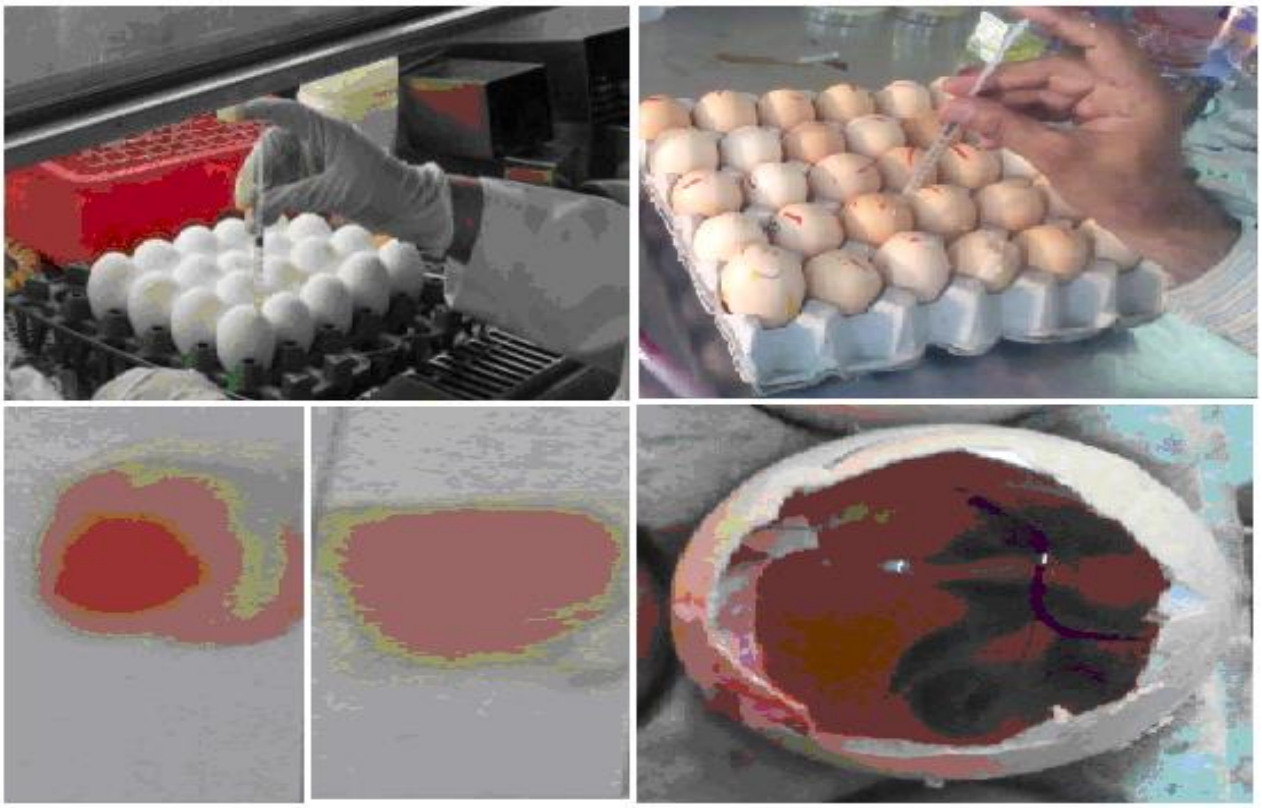

Fig. 1: Embryo inoculation, harvesting of chorioallantoic fluid and spot hemagglutination test

activity of *A. membranaceus* polysaccharides against Human simplex virus type-1 had been reported by Zhi-jie *et al*. (2003). Min *et al*. (2012) suggested the use of *Astragali aadi* as treatment option in different viral infections on the basis of antiviral activity of plant against experimental viruses. Similarly, Yuhao *et al*. (2001) reported activity of Astragalus polysaccharide, Epimedium polysaccharide and Epimedium flavones against NDV using chick embryo fibroblast. Dose dependent antivirus activity especially on capsid proteins expression of EBV by roots of *A. membranaceus* was determined by Xie *et al*. (2011). Higher efficacy of polysaccharides with no toxicity was observed by Liu *et al*. (2003) Human immuno deficiency virus (HIV) by Astragalus. Effectiveness of different Chinese herbal preparations was documented by Zhi-jie *et al*. (2003) while working on Herpes simplex virus using cell culture model. Shaukat *et al*. (2011) investigated that among the herbs, soya bean, green tea, catechin and opuntia species had antiviral activity against influenza virus. Three different concentrations (2,4 and 8%) of each extract and amantadine HCl were used through chorioallantoic route in 10-day old live chicken embryos. In case of amantadine HCl, out of 50, 500 and 1000µg/mL concentrations, only

500µg/mL was found to be an ideal concentration, as in addition to stopping the virus growth. It also did not kill the embryos. In case of *Opuntia dellinii* all the 3 concentrations used were not toxic for embryos, but antiviral effect was observed only at 4 and 8g/100mL concentrations. Green tea extract was found to be effective against AIV only at 8g/100mL concentration with no damage to chick embryos. Polysaccharides of *Astragalus* were trialed on dogs having immune suppression using different doses by Qiua *et al*. (2010) observing effects on cytokines and cells of immunity. Polysaccharides palyed positive role in boosting of immune system of treated dogs, reduction of clinical signs and improvement of health status. Dose dependent potential of polysaccharides was reported as immune booster. Different bioactive molecules of plants had activity against different viruses.

CONCLUSION

It was concluded that aqueous and methanol extracts of *A. membrananceus* can safely be used as therapy of infections caused by AI virus in poultry. However, further studies are required to investigate actual active ingredient of *A. membrananceus* having antiviral activity.

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