Comparative study to evaluate the anti-viral efficacy of *Glycyrrhiza glabra* extract and ribavirin against the Newcastle disease virus

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ABSTRACT

Background: The Newcastle disease represents as one of the most infectious viral disease, which afflicts almost every species of the birds. The causative agent of the disease is a single-stranded RNA virus with rapid replication capability. **Objective:** This study was performed to evaluate the comparative anti-viral efficacy and toxicity of *Glycyrrhiza glabra* aqueous extract and ribavirin against the Newcastle disease virus. **Materials and Methods:** The embryonated eggs were divided into six groups (A, B, C, D, E and F). Groups A, B, C, and D were further subdivided into three subgroups. The virus was identified by hemagglutination inhibition test. Spot hemagglutination test and viability of embryos were also evaluated. Three different concentrations i-e., 30 mg/100 ml, 60 mg/100 ml, and 120 mg/100 ml of the *Glycyrrhiza* aqueous extract and 10 µg/ml, 20 µg/ml, and 40 µg/ml ribavirin in deionized water were evaluated for their toxicity and anti-viral activity in the embryonated eggs. **Results:** 60 mg/100 ml concentration of *Glycyrrhiza* extract did not produce any toxicity in the embryonated eggs and showed anti-viral activity against the virus. Similarly, 20 µg/ml ribavirin was non-toxic in the embryonated eggs and contained anti-viral activity. **Conclusion:** It may conclude from the presented study that 60 mg/100 ml *Glycyrrhiza* extract inhibits replication of Newcastle disease virus and is non-toxic in the embryonated eggs. So, *Glycyrrhiza glabra* extract may be further evaluated in future to determine the potentially active compounds for their anti-viral activity against Newcastle disease virus. Furthermore, the mechanism of action of these active phytochemicals as an antiviral agent would be helpful to elucidate the pathogenesis of the disease.

Key words: Anti-viral agents, embryonated eggs, *Glycyrrhiza glabra,* Newcastle disease virus, Ribavirin

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INTRODUCTION

Newcastle disease (ND) is a highly contagious disease, which affects almost all species of the birds. It was first recognized in Indonesia and England in 1926,[1] and the disease virus has a worldwide prevalence.[2] The disease is caused by a single-stranded, enveloped, non-segmented RNA virus, which resembles in genome configuration to Avian Paramyxoviridae serotype-1 (APMV-1) of genus *Avulavirus* and the family Paramyxoviridae.[3] The genome of the Newcastle disease virus (NDV) is about 15.0 kb (kilo base) long[4] and encodes for six structural proteins in the order 3'-NP-P-M-F-HN-L-'5 respectively.[5] It is believed that fusion (F) protein is a major determinant of the virulence.[6]

Medicinal plants have been used all over the world for their therapeutic benefits, although their use remained restricted to China, India, Japan, Pakistan, Sri Lanka, Thailand and a number of African countries.[7] Similarly, the developed

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nations are also encouraging the use of natural medicinal products in their health care systems. Natural medicinal products in the forms of herbs have been commercially added in the dietary supplement industry as well as in holistic medicine in the United States. It has been estimated that one-third person in the United States has tried some form of natural medicine at least once.[8]

The traditional sources for the use of *Glycyrrhiza* species as an herbal medicine are reported in ancient manuscripts from China, India, and Greece. Its use for symptoms of viral respiratory tract infections and hepatitis has been documented by a number of researchers. Randomized controlled trials of the *Glycyrrhiza glabra* derived compound "glycyrrhizin," and its derivatives showed reduced hepatocellular damage in chronic hepatitis B- and C-infected patients. In hepatic cirrhosis induced by hepatitis C virus, the risk to develop hepatocellular carcinoma was reduced in those infected patients who administered with glycyrrhizin.[9] Glycyrrhizin (licorice root extract) has anti-inflammatory and antioxidant activities. Glycyrrhizin inhibits $CD4^+$ T-cell and tumor necrosis factor (TNF) - mediated cytotoxicity.[10] Glycyrrhizin has a membrane stabilizing effect[11] and also stimulates endogenous production of interferon.[12] 18-β glycyrrhetinic acid, an active constituent of glycyrrhizic acid, shows anti-viral activity against a number of DNA and RNA viruses, possibly due to activation of nuclear factor (NF-κB and induction of IL-8 secretion).[13]

Ribavirin is a nucleoside analog (also known as a nucleoside reverse transcriptase inhibitor), broad-spectrum anti-viral drug, which demonstrates anti-viral activity against a wide range of RNA and DNA viruses, including the hepatitis B, C, and retroviruses.[14] The drug's exact mechanism of action is still unclear; however, it is proposed that after phosphorylation into the cell, ribavirin inhibits inosine 5'-monophosphate dehydrogenase (IMPDH).[15] IMPDH inhibitors like ribavirin decrease the intracellular synthesis and storage of "guanine," a nucleotide base essential for DNA and RNA replication, consequently inhibiting viral replication.[16] The ribavirin pharmacokinetic profile, preclinical toxicity, safety, and clinical efficacy studies are well documented. The studies also show the use of ribavirin to treat respiratory syncytial virus infection in infants and young children and to treat influenza A and B virus infections in young adults.[17] Ribavirin aerosol has been used successfully to treat respiratory syncytial virus and para-influenza virus infection of immunodeficient children.[18] The aim of the current study was to investigate the efficacy of plant extract *Glycyrrhiza glabra* as an anti-viral agent against the Newcastle disease virus and to compare the efficacy of Ribavirin and *Glycyrrhiza* for the chemotherapy and prophylaxis of Newcastle disease virus.

MATERIALS AND METHODS

Embryonated eggs of 9th to 10th days were obtained from Hi-Tech Laboratories Pvt. Ltd. and were placed in WTO Laboratory. Live and dead eggs were separated by candling. A total of 90 eggs was collected and incubated at 37°C temperature in an egg incubator (70% humidity). The eggs were divided into six groups (A, B, C, D, E, and F). Groups A, B, C, and D were further divided into three subgroups.

Experimental procedure

Group-A1, A2, and A3 were designed to evaluate the anti-viral activity of *Glycyrrhiza*. Group-A1 contains 4HA virus with antibiotics (i-e., Penicillin 500000 IU/ml, Gentamycin 100 mg/ml, Streptomycin 500 mg/ml, and Amphotericin B 250 μg/ml) +2X (30 mg/100 ml) aqueous *Glycyrrhiza* extract. Group-A2 contains 4HA virus with antibiotics + 2X (60 mg/100 ml) aqueous *Glycyrrhiza* extract. Group-A3 contains 4HA virus with antibiotics + 2X (120 mg/100 ml) aqueous *Glycyrrhiza* extract. While groups-B-1, B-2, and B-3 were designed to evaluate the toxicity of *Glycyrrhiza*. Group-B-1 contains normal saline + 2X (30 mg/100 ml) aqueous *Glycyrrhiza* extract. Group-B2 contains normal saline + 2X (60 mg/100 ml) aqueous *Glycyrrhiza* extract. Group-B3 contains normal saline + 2X (120 mg/100 ml) aqueous *Glycyrrhiza* extract.

Groups-C1, C2, and C3 were designed to evaluate the anti-viral activity of Ribavirin.

Group-C-1 contains 4HA virus with antibiotics (i-e., Penicillin 500000 IU/ml, Gentamycin 100 mg/ml, Streptomycin 500 mg/ml, and Amphotericin B 250 μg/ml) +2X (10 μg/ml) aqueous solution of Ribavarin. Group-C2 contains 4HA virus with antibiotics + 2X (20 μg/100 ml) aqueous solution of Ribavarin. Group-C3 contains 4HA virus with antibiotics + 2X (40 μg/100 ml) aqueous solution of Ribavirin. Groups-D1, D2, and D3 were designed to evaluate the toxicity of Ribavarin. Group-D1 contains normal saline + 2X (10 μg/ml) aqueous solution of Ribavarin. Group-D2 contains normal saline + 2X (20 μg/ml) aqueous solution of Ribavirin. Group-D3 contains normal saline + 2X (40 μg/100 ml) aqueous solution of Ribavirin.

Group-E was kept as negative control, in which only normal saline was inoculated.

Group-F was kept as a positive control, in which only virus with normal saline was inoculated. 0.1 ml from each of the groups was inoculated into respective groups of embryonated eggs. The experiment was performed to evaluate the anti-viral effect as well as the toxicity of *Glycyrrhiza* extract (Root) and Ribavirin.

Glycyrrhiza extraction

Collection of plant material

The plant was obtained from the botanical gardens of Govt. College University, Lahore, Pakistan and ground to form a paste and was dried for overnight in Desiccators (MILLIPORE Desiccators).

Maceration

One reagent bottle of 500 ml capacity was used for maceration. Five-hundred ml of solvent i.e. deionized distilled water was taken with a bottle for *Glycyrrhiza*.

Five-hundred grams of *Glycyrrhiza* powder was weighed accurately using a digital balance and added in the bottle. The bottle was placed in a vibrator for 24 hours, and the plant material was allowed to macerate. The extract was filtered using Whatmann's filter paper. The water extract was subjected to Syringe Filtration, using filters of 0.2 μm size in safety cabinets.

Bacterial contamination test

One ml of extract was poured on a nutrient agar plate. The plate was left undisturbed for 2 minutes and after that, the excess of the extract was discarded from the plate. The plate was incubated overnight, and the growth of bacteria was observed on the plate. The aqueous extract of the plant did not show any growth of the bacteria.

Drying of the herbal extract

The filtrate was kept at 40°C for 90 hours in the incubator for drying. The extract (powder) was concentrated and weighed for *Glycyrrhiza*. After weighing, different aqueous experimental concentrations of *Glycyrrhiza* (15 mg/100 ml, 30 mg/100 ml and 60 mg/100 ml) were prepared accordingly to evaluate its toxicity and anti-viral activity.

Ribavirin

Four-hundred mg of ribavirin ("Variba®" of Bosch pharmaceutical Pvt. Limited) was mixed into 200 ml of deionized distilled water. From this aqueous solution, different experimental concentrations of ribavirin were prepared (5 μg/ml, 10 μg/ml, and 20 μg/ml) accordingly to evaluate its toxicity and anti-viral activity.

Source of virus

The Newcastle disease virus was obtained from Department of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan.

The virus was identified by Hemagglutination inhibition test (HI). The test was performed to estimate the 4HA activity of the test virus according to the method described by Allan and Gough, 1974.[19] The viability of the embryo in embryonated eggs i.e. live and dead embryo was evaluated by candling.

The air sacs and the heads of the embryos were demarcated by candling by using led pencils. Inoculum virus and virus plus a drug mixture were prepared by admixing 4HA suspension of ND virus along with the tested antibiotic and anti-fungal drug concentration (Penicillin 500000 IU/ml, Gentamycin 100 mg/ml, Streptomycin 500 mg/ml, and Amphotericin B 250 μg/ml).

The experimental groups, which were designed for the evaluation of toxicity and anti-viral activity of *Glycyrrhiza* extract and Ribavirin as well as the control groups, were inoculated with respective inoculums by making a pinpoint hole. The holes were sealed by using wax. The eggs were positioned with broadened ends upside and incubated for 72 hours with frequent candling after every 24 hours. The dead embryonated eggs were separated and were kept in the refrigerator at 4°C to 8°C till the final evaluation. After 72 hours, the groups designed for the anti-viral activity were checked for the replication of ND virus by means of spot Hemagglutination tests, whereas the groups designed for toxicity were checked for the viability of the embryo by candling and by opening the embryonated eggs.

The spot hemagglutination test was performed by taking one drop of allanto amniotic fluid from each egg and one drop of the chick RBCs suspension on slides. The slide which showed agglutination was considered as positive, while the slide without any agglutination was considered as negative.

RESULTS

The presence or absence of the virus was confirmed by using the hemagglutination property of the Newcastle disease virus. The *Glycyrrhiza* aqueous extract concentration 30 mg/100 ml failed to stop the replication of virus, while 60 mg/100 ml and 120 mg/100 ml inoculated embryonated egg's fluid showed absence of HA (Hemagglutination) activity. On the other hand, 30 mg/100 ml and 60 mg/100 ml concentrations did not cause the death of the embryo, whereas embryos in eggs inoculated with 120 mg/100 ml died, which indicate that 30 mg/100 ml and 60 mg/100 ml were non-toxic, whereas 120 mg/100 ml was a toxic drug concentration. Finally, it may conclude that the 60 mg/100 ml aqueous *Glycyrrhiza* extract is non-toxic and has anti-viral activity as shown in the Table 1.

For ribavirin 400 mg, the presence and absence of virus were verified by using hemagglutination property of Newcastle disease virus. The first drug concentration i.e., 10 μg/ml did not stop the replication of the virus, while 20 μg/100 ml and 40 μg/100 ml concentration showed no hemagglutination activity in inoculated embryonated

eggs. 10 μg/ml and 20 μg/ml concentrations did not cause the death of the embryo. On the other hand, embryos in eggs inoculated with 40 μg/ml died, which indicate that 20 μg/ml was non-toxic drug concentration while 40 μg/ml concentration was toxic. Finally, it may conclude that the drug concentration 20 μg/ml is non-toxic and has anti-viral activity, as shown in Table 2.

The Table 3 shows the comparison of anti-viral activity of *Glycyrrhiza glabra* extract and ribavirin at different drug concentrations.

DISCUSSION

ND virus was propagated in 9 to 10 days old embryonated eggs. The ND virus was identified by serological test known as a hemaagglutination inhibition test. In this test, the HA receptors on the ND virus were neutralized by specific antibodies, due to which ND virus failed to cause the hemagglutination of chicken RBC_S. This was in an agreement with the findings of Brugh *et al.*[20] and Rizwana *et al.*[21] who described that ND virus can be confirmed by hemagglutination inhibition activity along with the means death time (MDT), interacerebral pathogenicity index (ICPI), and an intravenous pathogenicity index. The virus caused some lesions on the embryo, when propagated in the chicken embryonated eggs.

The anti-viral activity of *Glycyrrhiza glabra* extract is due to the presence of anti-viral agent known as glycyrrhizic acid, which is water-soluble. The glycyrrhizic acid acts on different levels of the ND viral infection including adsorption, penetration, transcription, translation, assembly and releasing from the host cell. These findings are in line to the Pompei *et al.*,[22] Naksahima,[23] Baba and Shageta,[24] Badam,[25] Utsunomiya,[26] Harada,[27] Cristina *et al.*,[9] and Shiuan *et al.*[28] In these studies, the researchers indicated that glycyrrhizic acid inhibits the virus infection by the reduction of membrane fluidity, by the production of Gama interferon, inhibition of phosphorylating enzymes and reduction in viral latency. Furthermore, Crance *et al.*[29] described in 2003 that glycyrrhizic acid is comparable to the ribavirin because this is not only less toxic than ribavirin but also as a potent anti-viral as ribavirin when he studied it in tissue cultures. The results of the presented study are in line with these arguments as it was found out that only higher doses can be proved toxic when used as an anti-viral drug [Table 1].

The toxicity and anti-viral screening of *Glycyrrhiza* extract were comparable with ribavirin, which is a standard,

Table 1: Toxicity and antiviral effect of *Glycyrrhiza glabra* extract against Newcastle disease virus

No. of eggs	Negative control (Normal saline) G		Positive control (Virus) H		Toxicity (Anti-viral agent+Normal saline) Viab.			Antiviral activity (Anti-viral agent+4 HA virus)					
	Viab.	HA activity	Viab.	HA activity	B1 30 mg/100 ml	B2 60 mg/100 ml	B3 120 mg/100 ml	A1 30 mg/100 ml		A2 60 mg/100 ml		A3 120 mg/100 ml	
								HA activity	Viab.	HA activity	Viab	HA Activity	Viab
1	Live	-ve	Dead	+ve	Live	Live	Dead	+ve	Live	-ve	Live	-ve	Dead
2	Live	-ve	Dead	+ve	Live	Live	Dead	+ve	Live	-ve	Live	-ve	Dead
3	Live	-ve	Dead	+ve	Live	Live	Dead	+ve	Live	-ve	Live	-ve	Dead
4	Live	-ve	Dead	+ve	Live	Live	Dead	+ve	Live	-ve	Live	-ve	Dead
5	Live	-ve	Dead	+ve	Live	Live	Dead	+ve	Live	-ve	Live	-ve	Dead

Viab=Viability; HA=Hemagglutination activity; +ve=Positive; -ve=Negative

Table 2: Toxicity and anti-viral effect of Ribavirin against Newcastle disease virus in embryonated eggs

No of eggs	G Normal Saline		H Virus		F (Anti-viral with antibiotics+Normal saline) Viab.			E (Anti-viral agent with antibiotics+4HA virus)					
	Viab.	HA activity	Viab.	HA activity	F1 10 μg/ml	F2 20 μg/ml	F3 40 μg/ml	E1 10 μg/100 ml		E2 20 μg/100 ml		E3 40 μg/100 ml	
								HA activity	Viab.	HA activity	Viab	HA activity	Viab
1	Live	-ve	Dead	+ve	Live	Live	Dead	+ve	Dead	-ve	Live	-ve	Dead
2	Live	-ve	Dead	+ve	Live	Live	Dead	+ve	Dead	-ve	Live	-ve	Dead
3	Live	-ve	Dead	+ve	Live	Live	Dead	+ve	Dead	-ve	Live	-ve	Dead
4	Live	-ve	Dead	+ve	Live	Live	Dead	+ve	Dead	-ve	Live	-ve	Dead
5	Live	-ve	Dead	+ve	Live	Live	Dead	+ve	Dead	-ve	Live	-ve	Dead

Viab=Viability; HA=Hemagglutination activity;+ve=Positive; -ve=Negativ

Table 3: Comparative efficacy of *Glycyrrhiza glabra* extract and Ribavirin against Newcastle disease virus in embryonated eggs

Anti-viral Agent	Conc. Used	Total eggs	HA activity	Dead	Total eggs	Live	HA activity	Dead	HA activity
Ribavarin	10 µg/ml	5	-ve	5	5	5	-	0	+ve
	20 µg/ml	5	-ve	0	5	5	-ve	0	-
	40 µg/ml	5	-	5	5	0	-	5	-ve
Extract of *Glycyrrhiza*	30 mg/100 ml	5	-ve	0	5	5	+ve	0	+ve
	60 mg/100 ml	5	-ve	0	5	5	-ve	0	-ve
	120 mg/100 ml	5	-ve	5	5	0	-ve	5	-ve
Negative (-) Control (Anti-viral agent+normal saline)	All the embryonated eggs in this group were alive with negative (-ve) HA activity.								
Positive (+) Control (Anti-viral agent+4HA virus)	All the embryonated eggs in this group were dead with positive (+ve) HA activity.								

+ve=Positive; -ve=Negative; Conc. =Concentration

recognized, and FDA-approved anti-viral agent [Table 3]. Ribavirin has been recently used for the treatment of hepatitis B, C, and Herpes Megalo viral infections. Ribavirin stops particularly the viral replication by inhibiting the mechanism of duplication of viral nucleic acid as after the penetration in a host cell, the virus gets the control of cellular metabolism and uses the cellular machinery for its own replication and virus assembly. These facts are supported by the findings of Fenton and Potter,[30] John,[31] Connor *et al.*,[32] Hultgren *et al.*,[33] Lucia *et al.*,[34] Robert *et al.*,[35] Fernandez *et al.*,[18] Marina *et al.*,[36] Ramos *et al.*,[37] and Wang *et al.*[38] Herbal extracts contain a large number of therapeutically active compounds, and these phytochemicals are a potential source of molecular drug design and development against a large number of infectious viral diseases. It may conclude here that the aqueous extract of *Glycyrrhiza* contains the anti-viral activity due to glycyrrhizic acid; however, further studies are needed to determine the exact role of the potentially active ingredient in the viral inhibition in Newcastle disease. Similarly, complementary studies about the role of the active ingredients of *Glycyrrhiza* extract and its mechanism of action will also be helpful to postulate the exact role of each active component in viral inhibition and pathogenesis of the disease in the near future. Equivocally, glycyrrhizic acid and ribavirin use in combination to inhibit viral replication may be studied in the future for synergistic therapeutic effects. Further studies are required to know the histological changes in the viral infected host cells with or without aqueous *Glycyrrhiza* extract.

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