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Full Length Article

Evaluation of *In Ovo* Antiviral Activities of Medicinal Flowers against Newcastle Disease Virus and Avian Influenza Virus

Iqra Nazir, Masood Rabbani*, Ali Ahmad Sheikh, Muhammad Ilyas Riaz, Sohail Raza, Qurat ul Ain, Arfat Yousaf Shaheen and Aleena Kokab

Institute of Microbiology, University of Veterinary and Animal Sciences, Sheikh Abdul Qadir Jelani Road, Lahore 54000, Pakistan
*For correspondence: mrabbani@uvas.edu.pk
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Abstract

In Pakistan, the poultry industry is one of the rapidly growing industries. Due to lack of biosecurity measures, this is affected by some important infectious agents such as Avian Influenza virus (H_9N_2) and Newcastle disease virus (NDV) results in a huge economic loss. So, to control these losses discovery of new anti-viral drugs required to bring into line to fight against these infections. It is a general perception that the active components of medicinal plants have effective results against various infections like the influenza virus. The current therapeutic facilities need to be improved by investigating new antiviral drugs from natural resources to fight against viral infections. The present study was conducted on ethanolic extracts of seven different flowers to examine their antiviral activity against NDV and H_9N_2 *in ovo* using chicken embryonated egg inoculation. The spot agglutination and hemagglutination tests showed inhibitory effects of *Rosa damascena* Miller, *Achillea millefolium, Woodfordia fruticosa* Kurtz and *Bombax ceiba* L. against NDV as no agglutination observed. While the extracts of *Taxacum officianale* Weber, *Hyssopus officianalis* L. and *Chrysanthemum cinerafolium* (Trevis.) Vis. showed positive results for both spot agglutination and hemagglutination assay against NDV. However, both spot agglutination and hemagglutination assay showed inhibitory effect of all the flowers extracts against H_9N_2. The bioactive components such as alkaloids, ethers, terpenoids, *etc*. of each flower were analyzed through Gas chromatography mass spectrometry (GC-MS). The current results revealed that ethanolic extracts of these flowers possess strong antiviral activity because of their active ingredients. These ingredients should be isolated, commercialized and used for therapeutic purpose.

Keywords: Antiviral; Hemagglutination; Bioactive; GC-MS

Introduction

In Asia, medicinal plants are abundantly found to play a key role by providing valuable products for medicine. Approximately 75–80% of people depend upon drugs prepared from these plants to cure various viral diseases related to human and animals globally (Amber *et al*. 2017). Medicinal plants are relevant source to bring out innovative ideas in the research field regarding pharmacology with minimum side effects. Exploring new herbs having antiviral activity becomes limited because identifying antiviral ingredients from plants needs separation techniques that were lacked. Plants with broad-spectrum antiviral activity against emerging viruses of infectious diseases were screened (Mukhtar *et al*. 2008). In the past few years, synthetic drugs replaced by numerous herbal products resulted in harmful effects. This led to reviving the scientist's interest to medicinal products which could not be even replaced by modern chemistry. These plants are a rich source of bioactive ingredients with characteristics of antiviral potential and strong efficacy (Akram *et al.* 2018).

Newcastle disease is considered an economically major threat to the poultry industry. This virus belongs to the *Paramyxoviridae* family cause hemorrhagic intestinal lesions, respiratory distress, and impairs bird's growth. To control its infection lot of efforts have been put including vaccination but mutation within strain develop resistance among pathogen (Harazem *et al*. 2019). If chickens are infected with NDV it may results in extremely mild respiratory or enteric disease to severe systemic infection, cause high mortality, thus characterized by the rapid spread. Because of resistance development in the pathogen, there should be another source to control NDV. However, to prevent the replication of NDV or to decrease its severe effects on infected flocks different strategies are required (Dortmans *et al*. 2012; Miller *et al*. 2013).

Similarly, Influenza viruses' outbreaks remained a major threat worldwide with high mortality and morbidity. Influenza viruses are enveloped viruses having a genome in the form of segments. It is a negative sense, single-stranded RNA virus which belongs to Orthomyxoviridae family causes frequent epidemics almost every year (Tripathi *et al.*

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2020). A highly pathogenic avian influenza virus can infect and kill humans directly. It causes various infections such as respiratory tract problems or fatal systematic diseases in poultry. Different scientists have reported the antiviral potential of natural components against different viral infections in poultry birds and humans (Chang *et al*. 2013; Ahmed *et al*. 2017).

According to WHO, natural medicines are being used about three-quarters of the world population mainly different civilizations. Different countries have standardized their formulas for pharmacological products against various viral infections such as influenza virus and NDV. Although, synthetic chemical components are widely used in the modern era and the effectiveness of herbal products with the least side effects has been proved by scientists (Lee *et al*. 2020). However, previous studies remain limited about the antiviral activity of flowering parts of medicinal plants and their bioactive components. So, the present study was conducted on various medicinal flowers represents the strong antiviral activity of these against economically threatening viruses NDV and Avian Influenza virus.

Materials and Methods

Sample collection

100 g of dried flowers named *Achillea millefolium* L., *Bombax ceiba* L., *Chrysanthemum cinerafolium* (Trevis.) Vis., *Hyssopus officinalis* L., *Rosa damascena* Miller, *Taraxacum officinale* Weber and *Woodfordia fruticosa* Kurtz were purchased from local herbs market and transferred in a pre-labeled and clean zip-lock plastic bag to the Institute of Microbiology research lab. University of Veterinary and Animal Sciences, Lahore to determine the activity of the herbal extracts.

Preparation of ethanolic herbal plant extracts

Firstly, the Ethanolic extracts of medicinal flowers were prepared by grinding the flowers into fine amorphous powder. Briefly, 10 g of powder of each flower was soaked into 90 mL of 80 percent ethanol (1:10 w/v). After that, it was incubated for 2 days in a shaking incubator at 150 rpm at 37°C for the extraction of active compounds. Following incubation, the extracts were filtered through filter paper No.1 (Whatman, USA). The extracts were poured into glass Petri plates to dry at 40°C. Dried extract in crystal form was scratched and collected with the help of a spatula and further shifted in a clean microfuge tube (Eppendorf, Germany). The stock solution of each extract was prepared by using 10% dimethyl sulfoxide (DMSO) (Shaheen *et al*. 2015).

Virus samples

LaSota strain of NDV lyophilized tablet (1000 doses ampoule) was purchased from the market and Avian influenza virus ($H_9N_{2)}$ strain was taken from Quality operation laboratory UVAS. For further processing, samples were transferred to Microbiology research Lab. UVAS, Lahore, Pakistan.

Virus cultivation

Initially, reference strain of NDV (Lasota) and Avian influenza virus (H_9N_2) was propagated in 9 days old chick embryonated eggs that were purchased from the hatchery. To check the viability of eggs, candling was performed. The inoculum was prepared by mixing 1 mL viral suspension at room temperature (25°C) with antibiotic and antifungal agents. After that, 0.1 mL of prepared inoculum was injected into each of 10 eggs through the chorioallantoic sac route by using a sterile disposable syringe followed by incubation in an egg incubator at 37°C and 60–70 percent relative humidity. Out of all, two eggs served as a negative control contains only normal saline and added antibiotic. After incubation, the eggs were placed in refrigerator for 24 h and fluid was harvested (Grimes 2002).

Harvesting of fluid

Firstly, the eggs shells were disinfected by using 70% ethanol and the shells were removed using sterilized scissors. After that, chorioallantoic fluid was harvested and the spot agglutination test was performed by using 25 percent washed chicken RBCs to detect the presence of NDV and H_9N_2 (Grimes 2002). For further confirmation, hemagglutination test was performed and virus titers were found according to (Young *et al*. 2002) protocol. Harvested fluid was stored at -4°C for further testing.

Embryo Infectivity (EID50)

The embryo infective dose was calculated for each virus before evaluation of antiviral activity. Ten-fold dilutions of viruses were inoculated in groups of embryonated eggs as mentioned in Table 1 and 2. Each group contained four eggs. The embryos infectivity was observed up to 3 days according to Chollom *et al.* (2012). After that, the percentage index was calculated and the embryo infective dose was determined which is the titer of virus particles in a single dose causing 50% of embryos infection.

Antiviral activity of herbal extracts

Preparation of extracts dilutions: Dilutions of all the extracts were prepared in normal saline according to three different concentrations (200, 100 and 50 mg/mL) in microfuge tubes under sterile conditions (Raza *et al.* 2015).

Dilution of extracts and suspension of extracts and virus

Antiviral activity of flowers was determined by using three different concentrations of all extracts as C1, C2 and C3

Table 1: Embryo Infectious Dose 50 of NDV

Dilutions used	Infected (I)	Non-infected (NI)	I (A)	NI (B)	Total	Percentage
10^-1	4	0	9	0	9	9/9 x 100 = 100%
10^-2	4	0	5	0	5	5/5 x 100 = 100%
10^-3	1	3	1	3	4	1/4 x 100 = 25%
10^-4	0	4	0	7	7	0/7 x 100 = 0%
10^-5	0	4	0	11	11	0/11 x 100 = 0%
10^-6	0	4	0	15	15	0/15 x 100 = 0%
10^-7	0	4	0	19	19	0/19 x 100 = 0%
10^-8	0	4	0	23	23	0/23 x 100 = 0%

Calculation of the index:

Index= Percentage infected immediately above 50% - 50 ÷ Percentage infected at dilutions immediately above 50%-%infected at dilutions immediately below 50%

= (100% - 50%) ÷ (100%-25%)

= 50 ÷ 75

= 0.6

= 10^2.61 EID50 /0.1mL

= 10^3.6 EID50 /mL

Table 2: Embryo Infectious Dose 50 of H_9N_2

Dilutions used	Infected (I)	Non-infected (NI)	I (A)	NI (B)	Total	Percentage
10^-1	4	0	17	0	17	17/17 x 100 = 100%
10^-2	4	0	13	0	13	13/13 x 100 = 100%
10^-3	4	0	9	0	9	9/9 x 100 = 100%
10^-4	2	2	5	2	7	5/7 x 100 = 71%
10^-5	1	3	3	5	8	3/8 x 100 = 37.5%
10^-6	1	3	2	8	10	2/10 x 100 = 20%
10^-7	1	3	1	11	12	1/12 x 100 = 8%
10^-8	0	4	0	15	15	0/15 x 100 = 0%

Calculation of the index:

Index = Percentage infected immediately above 50% - 50 ÷ Percentage infected at dilutions immediately above 50%-%infected at dilutions immediately below 50%

= (71% - 50%) ÷ (71%-37%)

= 21 ÷ 34

= 0.61

= 10^4.61 EID50 /0.1 mL

= 10^5.6 EID50 /mL

against both NDV and H_9N_2. Antibiotics and antifungal agents were added in all suspensions and 4HA virus concentration was used for inoculum. The prepared virus/extract suspensions in ratio of 1:1 were kept at 37°C for 1 h (Suriani *et al.* 2015).

Evaluation of *in ovo* antiviral activity

To perform *in ovo* antiviral activity, total seven embryonated chicken eggs (ECE) groups were made having four eggs in each. Three groups were made according to concentrations C1, C2, C3. Three groups served as a negative control, contained 10% DMSO, pure extracts and normal saline while one group as positive control contained 4HA virus for each. Firstly, the viability of 9 days old ECE was observed and a hole was made above the air sac for inoculation. 0.1 mL of each concentration of inoculum was injected through the allantoic sac route into eggs with the help of a sterile syringe (0.1 cc) and a hole was sealed by sterile molten wax. The eggs were then incubated at 37°C. After 24 h, the embryonated eggs that died because of mechanical injury or microbial contamination discarded for NDV but for H_9N_2, the eggs died were placed in the refrigerator for chilling because it might be due to H_9N_2 pathogenicity. After 48 h of post-inoculation the eggs were placed in the refrigerator overnight for chilling. The chorioallantoic fluid was harvested to perform spot agglutination and hemagglutination test (Murakawa *et al*. 2003).

Viability and antiviral efficacy

Viability of harvested fluid and antiviral efficacy of different concentrations of extracts against NDV and H_9N_2 was checked by spot agglutination test and further confirmed by hemagglutination test. The harvested fluid was stored at -20°C in sterile Eppendorf (1.5 mL).

Gas chromatography mass spectrometry (GC-MS)

The ethanolic extract of all flowers in liquid form was sent to the Central laboratory complex (CLC) laboratory UVAS, Ravi campus for analysis of all the chemical and organic components in them. GCMS equipment used was of Agilant technologies, 7890B GC system, 5977B MSD MS system. The concentration and ratio of extracts were determined through this system. Molecules were separated based on volatility and polarity. Gas molecules used as mobile phase in it while column act as stationary phase and retention time of molecules was found by the detector. The results were shown in the form of peaks on the graph and abundance of components indicated by percentage area as mentioned in Table 5.

Statistical analysis

The data was examined by using statistic package for social sciences (SPSS) version 22.0. One way analysis of variance was applied and $P < 0.05$ value was taken as significant.

Table 3: Antiviral activity of flowers extracts against NDV

Extracts	Concentrations			Spot agglutination	Haemagglutination
	C1	C2	C3		
R. damascena	1:8	1:4	1:2	-ve	Bead formation
A. millefolium	1:8	1:4	1:2	-ve	Bead formation
B. ceiba	1:8	1:4	1:2	-ve	Bead formation
T. officianale	1:8	1:4	1:2	+ve	1:128
W. fruticosa	1:8	1:4	1:2	-ve	Bead formation
H. officianalis	1:8	1:4	1:2	+ve	1:128
C. cinerafolium	1:8	1:4	1:2	+ve	1:128

Table 4: Antiviral activity of flowers extracts against H_9N_2

Extracts	Concentrations			Spot agglutination	Haemagglutination
	C1	C2	C3		
R. damascena	1:8	1:4	1:2	-ve	Bead formation
A. millefolium	1:8	1:4	1:2	-ve	Bead formation
B. ceiba	1:8	1:4	1:2	-ve	Bead formation
T. officianale	1:8	1:4	1:2	-ve	Bead formation
W. fruticosa	1:8	1:4	1:2	-ve	Bead formation
H. officianalis	1:8	1:4	1:2	-ve	Bead formation
C. cinerafolium	1:8	1:4	1:2	-ve	Bead formation

Table 5: Spectral analysis of ethanolic extracts of Medicinal flowers

Flowers	Compound	Retention time (RT)	Area (%)
W. fruticosa	3-heptanol, 2 methyl-	6.165	21.87
T. officinale	Cyclopentanone, 2-methyl-	8.345	12.47
H. officinalis	3-butene-1-o1, 2-methyl	6.164	20.42
C. cinerarifolium	cyclopentanol	6.169	25.72
R. damascene	4-cyclopentene-1,3-diol, trans-	6.171	25.52
B. ceiba	Docosanoic acid	15.854	90.04
A. millefolium	5-Acetoxymethyl-2,6,10-trimethyl-2,9-undecadiene-6-ol	6.164	19.22

Results

According to the designed study, flower extracts were used to determine their antiviral activity by spot agglutination and hemaggltination test. Spot agglutination test for antiviral activity of extracts *Ro. damascena, A. millefolium, W. fruticosa* and *B. ceiba* against NDV was found negative as no agglutination occurs. For further confirmation hemagglutination test was performed against these extracts showed bead formation (tear-shaped on tilt) confirmed the inhibition of NDV. While the remaining extracts *Taxacum officianale* Weber, *H. officianalis, C. cinerafolium* showed positive results for both spot agglutination and hemagglutination assay indicated that these extracts did not effect on virus inhibition (Table 3). Multiple comparisons of statistical analysis, for NDV showed mean significant difference with $P < 0.05$ value of *W. fruticosa* extracts *A. millefolium, B. melabaricum, H. officianalis, C. cinerafolium*. For the antiviral activity of all the extracts *R. damascena, A. millefolium, W. fruticosa* and *B. ceiba , T. officianale, H. officianalis, C. cinerafolium* against H_9N_2 showed negative results for both spot agglutination and hemagglutination test indicates that these extracts strongly inhibited virus (Table 4). Multiple comparisons of statistical analysis, for H_9N_2 showed mean significant difference with $P < 0.05$ value of *W. fruticosa* extracts *A. millefolium, B. melabaricum, H. officianalis, C. cinerafolium, T. officianale* and *R. damascena*. The ethanolic extracts of all the flowers were analyzed through GC-MS and their active chemical constituents were determined. Spectral analysis of the ethanolic extract of *B. ceiba* (Gul-e-Simbal) contained seven components and among them, Docosanoic acid was the most abundant component with 90.04 percentage while Octane, 4, 5-dimethyl found to had highest retention time (Fig. 1). Spectral analysis of ethanolic extract of *Taraxacum officinale Weber* (Zar-e-Gul) examined eighteen components and the most abundant was 1-heptene, 5-methyl with a percentage area of 17.02 among them (Table 5). The Retention time of Butanoic acid, 3-hydroxy- was 5.402 with first peak. Spectral analysis of the ethanolic extract of *R. damascene* (Gul-e-Surkh) contained ten components among them Cyclopentanone, 2-methyl- was a major component and its percentage area was 17.21 (Fig. 2). Hexane, 3-methyl with retention time 5.399 with the first peak. *C. cinerarifolium* (Gul-e-Dawoodi) analysis through GC-MS examined ten active components and Cyclopentanone, 2-methyl was most abundant among them with 17.38 percentage area and Butyl isopentyl with minimum retention time 5.401. Ethanolic extract of *H. officinalis* (Gul-e-Zoofa) examined ten chemical constituents and 3-butene-1-o1, 2-methyl was major among them 20.42 percentage area. 2-bromopropionic acid, 2 ethylhexy1 ester among them had a minimum retention time of 5.395. Spectral analysis of the ethanolic extract of *W. fruticosa* (gul-e-dhawa) found 3-heptanol, 2 methyl as a most abundant component among sixteen components with 21.87 percentage area and 3,7 dimethyloctylacetate with minimum retention time of 5.183 (Fig. 3). Spectral analysis of ethanolic extract of *A. millefolium* (Gul-e-Birnajaisf) showed 5-Acetoxymethyl-2, 6, 10-trimethyl-2,9-undecadiene-6-ol as a major compound having 19.22 percentage area among fifteen compounds while Bicyclo [1.1.0] butane-1-carboxylic acid had minimum retention time of 5.175 (Fig. 4). GCMS analysis revealed the abundance of alcohol and ethers in all the flowers. Besides these, terpenoids, alkanes, and fatty acids were also found.

Discussion

Medicinal plants have remarkable antiviral effects at different stages of viral growth. Pharmacological products related to plants are being ranked highly for viral infections at this time. *W. fruticosa* flowers extract exhibit antiviral activity against enterovirus 71 but gallic acid extracted from its flowers were found to show more strong activity against

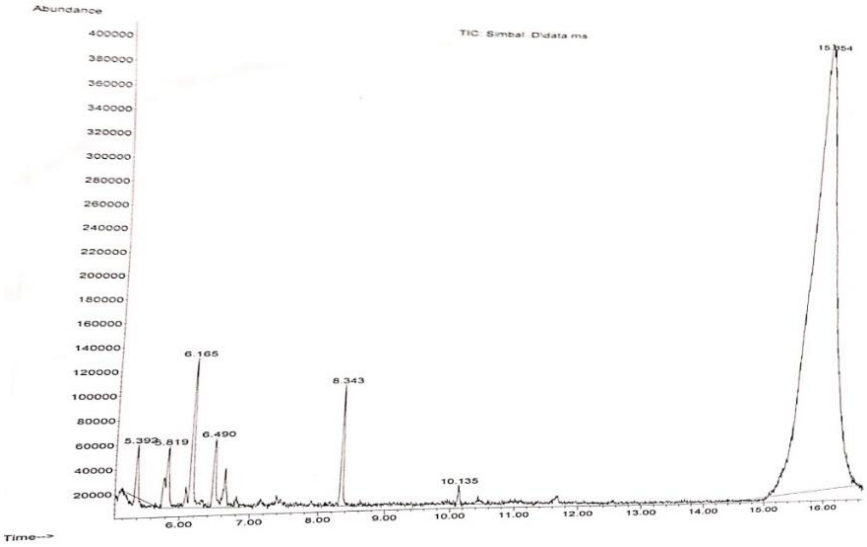

Fig. 1: Spectral analysis of *B. ceiba* flowers ethanolic extracts reveals different components in the form of peaks. Retention time of each component is indicated on X-axis and their abundance is shown on Y-axis

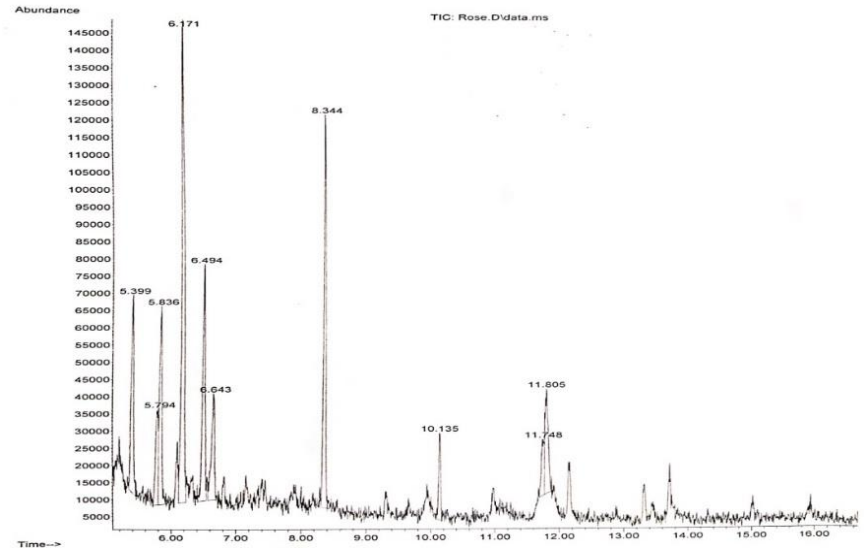

Fig. 2: Spectral analysis of *R. damascene* flowers ethanolic extracts reveals different components in the form of peaks. Retention time of each component is indicated on X-axis and their abundance is shown on Y-axis

EV-71 with an IC50 of 0.76 μg/mL at a concentration of 100 μg/mL (Choi *et al*. 2010). A recent study evaluated the antiviral activity of Gallic acid from *W. fruticosa* flowers against herpes simplex virus type 1 and human immunodeficiency virus (Kratz *et al*. 2008) comparable to the present study which showed strong antiviral activity of these flowers against NDV and H_9N_2 in chicken embryonated eggs. GC-MS examined sixteen chemical components of it and the most abundant among them was 3-heptanol, 2 methyl having 21.87 percentage areas with 6.165 RT. Retention time (RT) indicates the time taken by components to elute from column shown on chromatogram in the form of peaks.

A study conducted on the aqueous extract of *T. officinale* showed that it possesses antiviral activity against the influenza virus. The analysis was done by mini genome assay, real-time reverse transcription PCR found 0.625–5 mg/mL of *T. officinale* extracts ability to inhibit infections of PR8 or WSN viruses on human lung adenocarcinoma cell line (He *et al*. 2011) comparable with present study where it showed antiviral activity against H_9N_2 in contrast to NDV where it showed no inhibitory effect. The ethanolic extract of *Taraxacum officinale Weber* contained eighteen active components and 1-heptene, 5-methyl as most abundant among them while Butanoic acid, 3-hydroxy had minimum retention time.

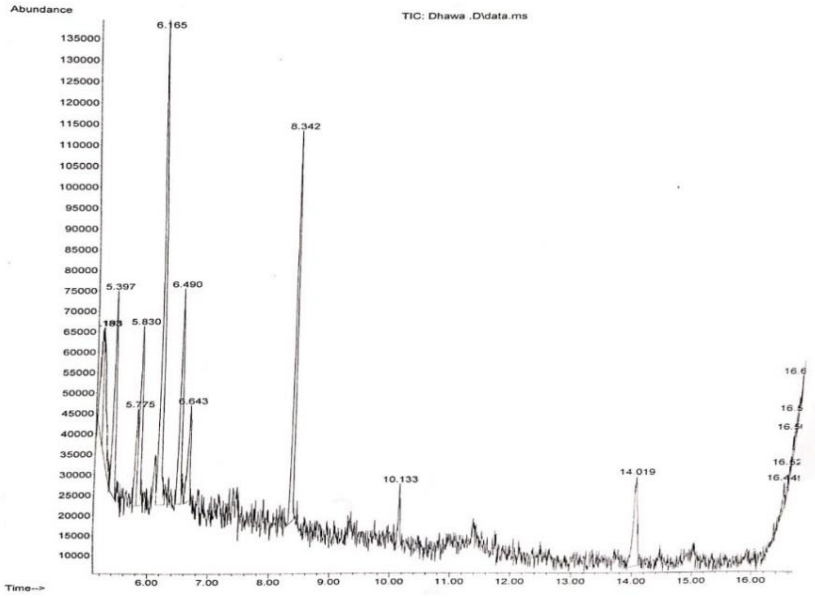

Fig. 3: Spectral analysis of *W. fruticosa* flowers ethanolic extracts reveals different components in the form of peaks. Retention time of each component is indicated on X-axis and their abundance is shown on Y-axis

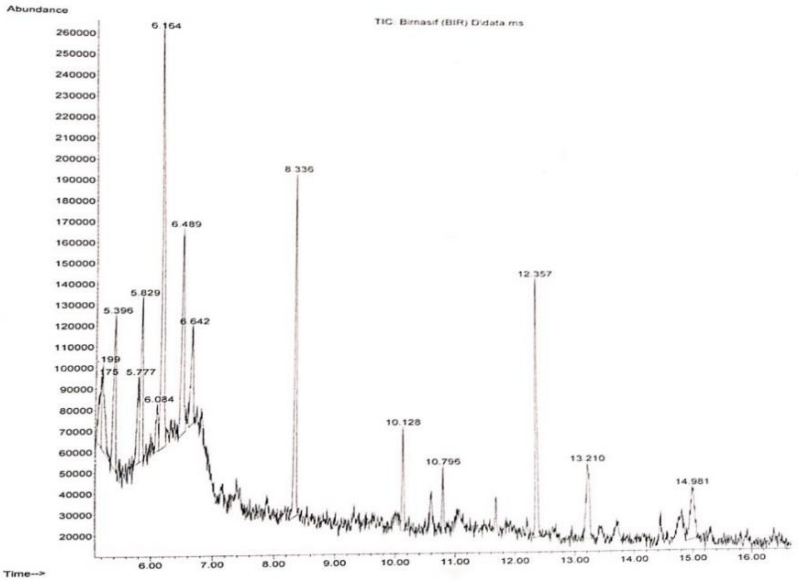

Fig. 4: Spectral analysis of *A. millefolium* flowers ethanolic extracts reveals different components in the form of peaks. Retention time of each component is indicated on X-axis and their abundance is shown on Y-axis

H. officinalis preparation are gaining much importance in food industries as well as in herbal remedies (Dragland *et al*. 2003; Jung *et al*. 2004; Lugasi *et al*. 2006). The extracts of *H. officinalis* contained tannins and some high molecular weight compounds which are unidentified as well as caffeic acid having strong antiviral activity against HIV. It might be used for the treatment of AIDS (Kreis *et al*. 1990). Essential oils and a lot of polyphenolic compounds are the main active ingredients of this plant according to biological and chemical aspects studied by the literature review (Benedec *et al*. 2003; Fathiazad and Hamedeyazdan 2011; Vlase *et al*. 2014). The Current study revealed the flowers extracts of *H. officinalis* with strong antiviral activity against H_9N_2 inhibited viral growth in embryonated eggs but against NDV, did not show any response. The Spectral analysis of *H. officinalis* flowers were evaluated which contained ten chemical constituents and 3-butene-1-o1, 2-methyl as abundant ingredient having 20.42 percentage areas. The study on *R. damascene* plant found many active constituents including anthocyanins, flavonoids, glycosides, and terpenes having beneficial

properties for human health. Petals of its flowers are rich in vitamin C and flavonoids. *In vitro* study showed its inhibitory effect on HIV infection with > 100 and 50 selective indices, respectively (Mahmood *et al*. 1996) relatable to the present study, it showed strong antiviral activity of *R. damascena* flowers against both NDV and H_9N_2. GCMS analysis of it found ten chemical constituents and 4-cyclopentene-1, 3-diol, trans was the most abundant.

The study of *B. melabaricum* flowers phenolic compounds showed its strong activity against RSV *in vitro* with a 50 μg/L IC50 value. All the compounds of ethanolic extracts were evaluated and three among them were considered as having antiviral activity. Flavonoid, glycoside, caffeoyl acid and kaempferol-3-O-β-D provide potent antiviral activity against RSV. Many other compounds also possess anti-RSV activity (Zhang *et al*. 2015) related to the present study showed antiviral activity in chicken embryonated eggs against avian influenza virus but in contrast to NDV having no inhibitory effect. The ethanolic extracts of *A. kellalensis* flowers possess anti-rotavirus activity *in vitro*. The Dose of 100 μg/mL extract of *A. kellalensis* was observed as the effective concentration of extracts. Anti-bovine rotavirus extracts of *A. kellalensis* exhibit potent antiviral activity and it could be because of phenolic acids (Rustaiyan *et al*. 1999; Si *et al*. 2006; Kwon *et al*. 2010), flavonoids (Bae *et al*. 2000) that are RNA synthesis blockers (Kwon *et al*. 2010). The study analyzed that in veterinary medicine, the use of this herb for treatment purposes will be effective (Taherkhani *et al*. 2013). In the present study, *A. millefolium* flowers extract also showed antiviral activity against avian influenza virus in contrast to NDV where it did not show any inhibitory effect. The ethanolic extract of *c. cinerafolium* found ten chemical components in its flowers with cyclopentanol as abundant. It showed antiviral activity against H_9N_2 but no response against NDV. ANOVA applied for multiple comparisons of statistical analysis in the present study for AIV showed mean significant difference with *P*-value < 0.05 of *W. fruticosa* extracts *A. millefolium, B. melabaricum, H. officianalis* and *C. cinerafolium*. The components identified though GCMS in these extracts includes ethers, fatty acids, flavonoids etc. However, further investigations are required about the activity of these bioactive components. The *in vitro* study on cell lines will be helpful to find out their particular mode of action.

Conclusion

The study conducted on seven different medicinal flower extracts showed their antiviral activity against H_9N_2 and NDV. All the extracts have significant antiviral potential with a $P < 0.05$ value for both viruses except *T. officianale* Weber, *H. officianalis* and *C. cinerafolium* against NDV. Furthermore, GCMS analysis examined several chemical ingredients in the form of different peaks. These active constituents have a significant role in the flower's antiviral activity. It is suggested to extracts these specific components for *in vitro* study so that can be used for therapeutic purpose and prophylactic measures in future. Moreover, the active products of these flowers should be isolated and commercialized for use in feed.

Author Contributions

MR, AAS, SR conceived and designed the experiments. IN and MIR executed the experiments and analyzed the study results. QA and AK helped in research work. AYS helped in writing the manuscript. IN wrote and edited the paper. All authors critically revised the manuscript for important intellectual contents and approved the final version.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable in this paper.

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