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Antiviral activity of crude extracts of *Eugenia jambolana* Lam. against highly pathogenic avian influenza (H5N1) virus

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Crude extracts of leaves and bark of *E. jambolana* were tested for antiviral activity against highly pathogenic avian influenza virus (H5N1) by CPE reduction assay in three different layouts to elucidate virucidal, post-exposure and pre-exposure antiviral activity of the extracts. The cold and hot aqueous extracts of bark and hot aqueous extract of leaves of *E. jambolana* showed significant virucidal activity (100% inhibition) which was further confirmed in virus yield reduction assay (~98 to 99% reduction) and by egg based *in ovo* assay. The selective index (CC_{50}/EC_{50}) of hot aqueous extract (248) and cold aqueous extract (43.5) of bark of *E. jambolana* showed their antiviral potential against H5N1 virus. The significant virucidal activity of leaves and bark of *E. jambolana* merits further investigation as it may provide alternative antiviral agent for managing avian influenza infections in poultry farms and potential avian-human transmission.

Keywords*:* Antiviral, Avian influenza, *Eugenia jambolana*, H5N1, Herbal, *In ovo*

Avian influenza (AI) is a highly contagious disease of poultry caused by type A influenza viruses of the family Orthomyxoviridae[1]. The rapid rate of spread and the high potential for genetic alterations of the virus has raised the spectre of widespread human infection and the possibility of a pandemic[2]. With emergence and multi-regional spread of AI, especially highly pathogenic avian influenza (HPAI) of H5 and H7 subtypes in poultry, the epizootic has alerted the world to the prospects of a potentially devastating human health challenge. The HPAI H5N1 subtype virus has caused disease outbreaks in poultry in several countries in Asia including India[3-6].

The role of antivirals is considered critical in preparedness for avian flu originated pandemic. Several at-risk nations and WHO have stored strategic stockpiles of antivirals especially oseltamivir to be used at the face of influenza pandemic. However, resistance to oseltamivir in the H5N1 subtype in Vietnam[7] and other human influenza A viruses[8] has become a cause for worry as far as pandemic preparedness is concerned. Therefore, the search for alternative antivirals that can effectively inhibit H5N1 or other influenza A viruses, or act in synergy with available antivirals, is an urgent need of the hour. Several novel antiviral agents that may be effective against influenza virus, specifically the H5N1 avian flu virus, are currently under development. Among these, plant-derived extracts have become focus of many studies due to their proven beneficial health effects in several disease problems[9].

Eugenia jambolana Lam. (Syn. *Syzygium cumini* Skeels or *Syzygnium jambolana* Dc) belonging to the family Myrtaceae, is a large evergreen tree up to 30 m. It is widely distributed throughout India, Sri Lanka-Malaya and Australia and known as *Jamun* in India[10]. The medicinal value of *E. jambolana* has been recognized in various system of traditional medication for the treatment of different diseases and ailments. In Ayurvedic system of medicine, its bark is used as decoction in chronic diarrhoea and dysentery, as gargle in sore throat, spongy gums, etc[11,12]. Juice of the tender leaves of *E. jambolana* is used to cure dysentery with bloody discharge[10]. Essential oil and crude extracts of *E. jambolana* leaves have been reported to possess antibacterial[13,14] and antiviral[15] properties. The present study has attempted to reveal

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antiviral properties of extracts of leaves as well as bark of *E. jambolana* against HPAI H5N1 virus in Madin Darby Canine Kidney (MDCK) cells (*in vitro*) and in chicken embryonated eggs (*in ovo*).

Materials and Methods

Extract preparation—The leaves and bark of *E. jambolana* were collected during July and August, 2008 from Western Uttar Pradesh belt of India. The plants were authenticated by Botanical Survey of India, Central National Herbarium, Govt. of India (Voucher specimen number CHN/1-1(174)/2007/Tech2/104). The methanolic, hydro-methanolic, hot aqueous and cold aqueous extracts of leaves and hot aqueous and cold aqueous extracts of bark were prepared using Soxlet apparatus (FOSS SOXTEC 2045, Analytical AB, Sweden). After drying, each extract was re-suspended in its respective extraction medium (w/v), centrifuged, filter-sterilised and stored as a stock of 100 mg/mL for further use.

Cells and virus stock—MDCK cells were propagated in Glasgow's Minimum Essential Medium (GMEM, Sigma, St. Louis, USA) supplemented with 10% fetal bovine serum (FBS) [Hyclone, Utah, USA]. The HPAI H5N1 virus (A/chicken/Navapur/Nandurbar/India/7972/2006) was obtained from the OIE reference lab for avian influenza at High Security Animal Disease Laboratory (HSADL) Bhopal and the work was carried out inside its BSL3+ containment laboratory. The virus was grown in MDCK cells and the cell-free virus supernatant was frozen (-70^{o}C) in aliquots until further use. The virus was titrated as $TCID_{50}$ (tissue culture infective dose 50%)[16] and as plaque forming units by plaque assay[17].

Cytotoxicity assays—The maximum non-toxic concentration (MNTC) of each extract and the drug controls, namely oseltamivir carboxylate (kindly gifted by Roche) and amantadine hydrochloride (Sigma, St. Louis, USA; cat. No. A1260) was determined based on cellular morphologic changes[18]. In brief, different concentrations of each extract (double dilutions of the stock of 100 mg/mL) were incubated in contact with confluent monolayer of MDCK cells in triplicate in 96-well plates for 4 days and the cells were observed under microscope every 24 h for visible morphological changes. The highest concentration of the extract without any cellular morphologic change up to 4 days in all the three replicates was considered as its MNTC. The extracts were tested by CPE reduction assay and virus yield reduction assay at their MNTCs.

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide a yellow tetrazole) assay was performed to evaluate the cytotoxic effects of extracts on MDCK cells[19,20] and to determine 50% cytotoxic concentration (CC_{50}). The cells were grown in 96-well tissue culture plates for 24 h followed by replacement of growth media with the serially diluted herbal extracts in GMEM medium and incubation of the cells for 48 h. The cell viability was evaluated by removal of the medium and addition of 25 µL MTT solution (5 mg/mL; Sigma, St. Louis, USA) to each well and incubation at 37°C for 4 h. After removal of the supernatant, 50 µL DMSO was added to dissolve formazan crystals and the mixture was incubated for 30 min. The optical density (OD) was measured at 540 nm in an ELISA reader (Tecan-sunrise). The CC_{50} of each extract was calculated by GraphPad Prism software as described earlier[17].

Cytopathic effect reduction assay—Each extract and the drug controls (amantadine and oseltamivir) were tested at MNTC for anti-viral activity against HPAI virus by CPE reduction assay[21] in three different layouts to elucidate virucidal, post-exposure and pre-exposure antiviral activity as described below in three independent experiments.

Virucidal activity: Equal volumes of the extracts at MNTC and virus were mixed and incubated at 37°C for 1 h. Mix (100 µL) was added to the confluent monolayer of MDCK cells (~2×10^4 cells/well) in 96-wells plate at MOI (multiplicity of infection) ~ 0.01. The plate was incubated at 37°C and the wells were observed under an inverted microscope for virus-induced CPE at 24, 48 and 72 h post-infection to record a score for each well on the basis of extent of CPE in the particular well (score 0 for 0% CPE, score 1 for 0–25% CPE, score 2 for 25–50% CPE, score 3 for 50–75% CPE and score 4 for 75–100% CPE). The cytotoxicity control (uninfected but extract-treated cells) at each treatment concentration, the cell control (uninfected untreated cells) and the virus control (infected but untreated) were kept in each plate throughout the test.

Post-exposure antiviral activity: Confluent monolayer of MDCK cells (~2×10^4 cells/ well) in 96-wells plate were incubated for 1 h with virus (100 µL vol/ well at MOI ~ 0.01) except in the 4 wells of cell control. The plate was washed thrice with plain GMEM after discarding virus inocula. Extract at its MNTC in plain

medium were added in triplicate to the wells and were incubated for 72 h. CPE scores were noted at 24, 48 and 72 h post-infection as described in virucidal layout, above. The virus control (virus + plain medium) and the standard drug controls (virus + drugs at their MNTCs) with oseltamivir and amantadine and the cell control (uninfected cells in plain medium) were included in the study.

Pre-exposure antiviral activity: The confluent monolayer of MDCK cells (~2×10^4 cells/well) in 96-wells plate cells were pre-incubated with different extracts at their MNTCs in triplicates for 1 h. The controls were kept as described above. The cells were washed once and then incubated for 1 h at 37°C with virus at MOI ~ 0.01. The virus inocula were discarded and the plate was washed thrice and overlaid with plain medium and incubated at 37°C in 5% CO_2 for 72 h. CPE scores were noted every 24 h intervals as described above.

Infectious virus yield reduction assay—The herbal extracts were further evaluated by infectious virus yield reduction by plaque assay. The antiviral effect of the extracts was assessed by its ability to reduce the number of PFUs from the untreated control samples[22].

Plaque assay was carried out in 6-well plates with confluent monolayer of MDCK cells (~1×10^5 cells/well) in three layouts as described in CPE reduction assay. In virucidal layout, extract-treated virus (at MOI ~ 0.01) was overlaid on cells and incubated for 1 h. In pre-exposure layout, the monolayer was pre-treated with extracts (at MNTC) for 1 h followed by addition of virus at MOI ~ 0.01 for 1 h. After washing thrice with pre-warmed GMEM, the monolayer in each well was overlaid with 2.5 mL of 1% agarose (Promega, V211A) supplemented with 5% serum in GMEM. In post-exposure layout, the untreated virus was overlaid (at MOI ~ 0.01) on the monolayer for 1 h. After washing with GMEM thrice, the wells were overlaid with 1% agarose overlay mixed with extracts (at MNTC). The plates were incubated at 37°C with 5% CO_2 for 48 h and stained and fixed with 0.1% crystal violet in 10% formalin (Sigma-F-A775) for 4 h at 37°C. Plaques were counted after washing and removing agarose. The mean titre of infectious virus was calculated in terms of pfu/ml from three independent experiments and the percent reduction in virus yield for each of the herbal extract was calculated as mentioned below:

$$\text{Virus yield reduction (\%)} = 1-\frac{\text{titre of treated virus (pfu/mL)}}{\text{titre of untreated virus control (pfu/mL)}}\times100$$

Dose-response study—Dose response assay was carried out by estimating viral load after treating the fixed amount of virus (200 pfu) with different dilutions of the selected extracts showing virucidal activity by plaque assay as described above. The minimal concentration of the extracts required to reduce the 50% of plaque number (EC_{50}) was calculated by interpolation of data from dose response curve using GraphPad Prism software. The selective index (SI) for each extract was calculated as ratio of CC_{50}/EC_{50}.

Haemagglutination inhibition (HI) assay—HI assay was employed to evaluate the effects of the selected herbal extracts on viral adsorption to chicken RBCs[17] with some modifications. Briefly, two-fold dilutions (0.025 mL) of the extracts and 4 haemagglutination units (HAU) of the virus (0.025 mL) were incubated for 1 h at 37ºC in 96-well plate followed by addition of 0.05 mL of 1% (v/v) chicken RBCs and reading after 40 min. To differentiate between the effects of extract on the RBCs, controls of each extract with only RBCs and no virus were also kept. Similarly, two-fold dilutions of vehicle (extraction medium) were incubated with RBCs and without virus as vehicle control. The results of HI were considered valid only if there was no effect of the extract or the vehicle on RBCs in absence of virus. The highest dilution of each extract showing inhibition of haemagglutination (HA) was taken as its HI titre.

In ovo antiviral activity—The extracts showing virucidal activity in previous experiments were tested by egg based assay for *in ovo* antiviral activity. Specific pathogen free embryonated chicken eggs (SPF-ECE) used in the experiment were obtained from the SPF facility of HSADL and were used between 9 to 11 days of incubation. The selected extracts of *E. jambolana* were tested in three independent experiments i.e. I, II and III with 3, 3 and 4 SPF-ECE respectively. 100 EID_{50} (100 µL) of extract treated virus (at MNTC), drug control (virus + amantadine) and the untreated virus control were inoculated in the allantoic cavity. The eggs were incubated at 37°C with 70% humidity for 96 h. The percent of infected eggs out of total 10 inoculated eggs and percent reduction in mean HA titre of the infected eggs was calculated for each extract.

Detection of phytochemical groups—Qualitative chemical analysis of the selected extracts was carried out to detect major phytochemical groups viz

reducing sugars, glycocides, flavanoids, proteins, sterols, resins, alkaloids, and tannins by standard methods[23].

Statistical analysis—Results are given as arithmetic mean values ± SD for three independent experiments. Student's unpaired *t test* was used to evaluate the difference between the test sample and untreated control. A *P* value of <0.05 was considered statistically significant.

Results and Discussion

Influenza virus continues to emerge and re-emerge and remains a major public health concern[24]. As an alternative to chemically synthesized antivirals such as amantadine[25] or oseltamivir[26], many plant extracts and purified substances have been tested and reported to have selective antiviral activities inhibiting influenza viruses[27,28]. As a continued approach for identifying antiviral substances of plant origin, the antiviral potential of crude extracts of leaves and bark of an indigenous tree *E. jambolana* have been tested against avian influenza virus (H5N1 subtype) in the present study which seems to be the first report on antiviral activity of *E. jambolana* against influenza virus.

Cytotoxicity of herbal or other drugs in respect of a target cell is an important criterion for its suitability for *in vitro* study as the antiviral drug should be effective against the virus without inducing cytotoxic effect. In the present study, the MNTC of the extracts varied from 0.0098-0.1563 mg/mL (Table 1). The concentrations above MNTC were toxic to the cells as evidenced by rounding, clumping and detachment from the well surface. The CC_{50} values estimated by MTT assay were used along with EC_{50} to calculate SI of each extract (Table 2).

The extracts of *E. jambolana* exhibited significant virucidal property (inhibition up to 100% with *P*<0.05) against H5N1 virus in CPE reduction assay (Table 3). In the virucidal assay, the complete inhibition of CPE up to 72 h PI by cold and hot aqueous extracts of bark (Fig. 1) and hot aqueous extract of leaves of *E. jambolana* was a significant finding of the present study revealing the antiviral potential of these extracts.

In the virucidal layout, reduction in the virus yield was maximum (~99.79%) in the virus treated with the hot aqueous extract of bark followed by cold aqueous extract of bark (99.58%), hot aqueous extract of leaves (98.95%) and cold aqueous extract of leaves (98.31%). The positive drug control, amantadine was found to reduce virus yield by 86% (Table 4). These results correlated well with the results in CPE reduction assay. The observation that crude herbal extracts of *E. jambolana* were more effective than amantadine may be due to synergistic effect of multiple ingredients present in the crude extracts in comparison to the purified drug control. The similar virucidal activity in both hot as well as cold extracts also suggests that the active component may be heat resistant. There was, however, no effect of extract on virus in the pre-exposure as well as the post exposure format (data not shown). The results indicate that extracts of *E. jambolana* may not have an effect on replication phase of the virus or effect on the cells to prevent entry of the virus but directly inactivate H5N1 influenza virus and might interfere with viral envelop or mask viral structures that are necessary for adsorption or entry into host cells.

The dose response study showed that the percent inhibition of plaque count decreased in a dose-dependent manner in all the four extracts studied (Fig. 2). In case of hot aqueous extract of bark, the end-point (0% PI) reached at a lower concentration (0.49 µg/mL) in comparison to cold aqueous extract

Table 1—Cytotoxicity of *E. jambolana* extracts on MDCK cells

Extract	MNTC (mg/mL)
Bark, hot aqueous extract	0.0781
cold aqueous extract	0.0781
Leaves, hot aqueous extract	0.0781
cold aqueous extract	0.1563
methanolic extract	0.0098
hydro-methanolic extract	0.0098
Amantadine	0.9×10^{-3}
Oseltamivir	2.2×10^{-2} µg/mL

Table 2—Antiviral (virucidal) efficacy of the *E. jambolana* extracts

[Values are mean ± SD of 3 independent experiments]

Extract	#CC_{50} mg/mL	†EC_{50} µg/mL	Selective index
Bark, hot aqueous extract	0.318±0.23	1.28±0.08	248
cold aqueous extract	0.378±0.24	8.69±0.66	43.5
Leaves, hot aqueous extract	0.12±0.05	15.22±2.11	8
cold aqueous extract	0.18±0.04	14.82±1.44	12

#CC_{50} represents the concentration of the extracts causing 50% cell toxicity relative to the control cells without extract.

†EC_{50} represents the concentration of extract necessary to reduce the plaque number by 50% relative to the control wells without test compounds, calculated from dose-response data of virucidal plaque reduction assay in MDCK cells

of bark (0.98 µg/mL), cold aqueous extract of leaves (7.81 µg/mL) and hot aqueous extract of leaves (3.91 µg/mL). The EC_{50} was least in hot aqueous extract of bark (1.28±0.08) in comparison to the other three extracts (Table 2). The SI of hot aqueous extract of bark was maximum (248) indicating a very good potency and wide margin of safety in comparison to other extracts (Table 2).

In the present study, phytochemical analysis of the extracts showed presence of tannin, coumarin, saponin and reducing sugars in hot and cold aqueous extracts of bark, alkaloids, tannin, coumarin, sterols and reducing sugars in hot and cold aqueous extracts of leaves. The leaves and bark of *E. jambolana* are reported to contain a number of polyphenols and flavonoids quercetin and myricetin[10]. Polyphenol rich extract from medicinal plant *Geranium sanguineum* L. was reported to have anti-influenza virus activity[29]. Flavonoid compounds are also known to inhibit both, the activity of neuraminidase and membrane fusion[27]. Catechins and other flavonoids present in the green tea have also been reported to inhibit haemagglutination as well as neuraminidase, affecting the viral RNA synthesis[17]. Though the active principle inhibiting the H5N1 virus in crude extracts have not been identified in the present study, it is possible that it could be any of these compounds or their combination. Although present antiviral research is aimed at identifying a specific molecule responsible for antiviral effect, the traditional system of medicine is based on whole plant parts or their crude extracts, because they are thought to contain synergistic elements that interacts with the causative agent as well as the host system to eliminate the agent. It is believed that studies with crude extracts of herbs, such as the present one, are as important as the ones which demonstrate antiviral effect of isolated molecules. Nevertheless, active principle in these extracts needs to be identified.

The extracts were also tested for their ability to inhibit HA in the present study, though the HI assay was found to be less suitable as antiviral assay than the plaque assay mainly due to the toxicity of the studied extracts to RBCs. However, there was still indication of inhibition of HA by hot and cold aqueous extracts of bark of *E. jambolana* at minimum concentration of 24 µg/mL concentration. The cold and hot aqueous extracts of leaves of *E. jambolana* did not show inhibition of HA (Table 5). Since the hot and cold aqueous extracts of bark of *E. jambolana* inhibited HA activity, it may be concluded that the active compound in these extracts may block entry of treated virus into the cells. However, the virucidal effect in these extracts was observed at much lower concentration (EC_{50} 1.28 and 8.69 µg/mL) suggesting an additional basis for their observed antiviral effect on H5N1 virus. Moreover,

Table 3—Screening of *E. jambolana* extracts for antiviral effect on H5N1 virus by CPE inhibition assay in MDCK cells

[Values are mean ± SD of 4 replicates. The figures in parenthesis are % inhibition from the virus control]

Extract[$]	CPE score (% inhibition)					
	Virucidal		Post-exposure		Pre-exposure	
	48 h	72 h	48 h	72 h	48 h	72 h
Virus control	1.75± 0.5	3.75± 0.5	3.0± 0	4.0± 0	3.0± 0	4.0± 0
Bark, hot aqueous extract	0±0 (100)*	0±0 (100)*	2.5±0.58 (17)	3.25±0.96 (19)	3.75±0.5 (-25)	4.0± 0 (0)
cold aqueous extract	0±0 (100)*	0±0 (100)*	2.5±0.58 (17)	4± 0 (0)	4.0± 0 (-33)	4.0± 0 (0)
Leaves, hot aqueous extract	0±0 (100)*	0±0 (100)*	2.5±0.58 (17)	0±0 (0)	3.0± 0 (0)	4.0± 0 (0)
cold aqueous extract	0±0 (100)*	2.25±0.50 (40)*	2.75±0.50 (8)	0±0 (0)	4.0± 0 (-33)	4.0± 0 (0)
methanolic extract	3.25±0.50 (-86)	4± 0 (-7)	3.25±0.50 (-8)	0±0 (0)	3.25± 0.5 (-8)	4.0± 0 (0)
hydro-methanolic extract	1.5± 0.82 (14)	3± 0.82 (20)	2.5±0.58 (17)	0±0 (0)	2.75± 0.5 (8)	4.0± 0 (0)
Amantadine	0.0± 0 (100)*	1.75± 0.5 (53)*	0.0± 0 (100)*	2.25± 0.5 (40)*	0.0± 0 (100)*	1.75± 0.5 (56)*
Oseltamivir	0.0± 0 (100)*	2.25±0.5 (40)*	0.0± 0 (100)*	1.75± 0.5 (53)*	1.0± 0 (67)*	4.0± 0 (0)

[$]The extracts and the control drug were tested at respective MNTC as shown in Table 1.
*Mean CPE score was significantly inhibited (P<0.05) from that of virus control.

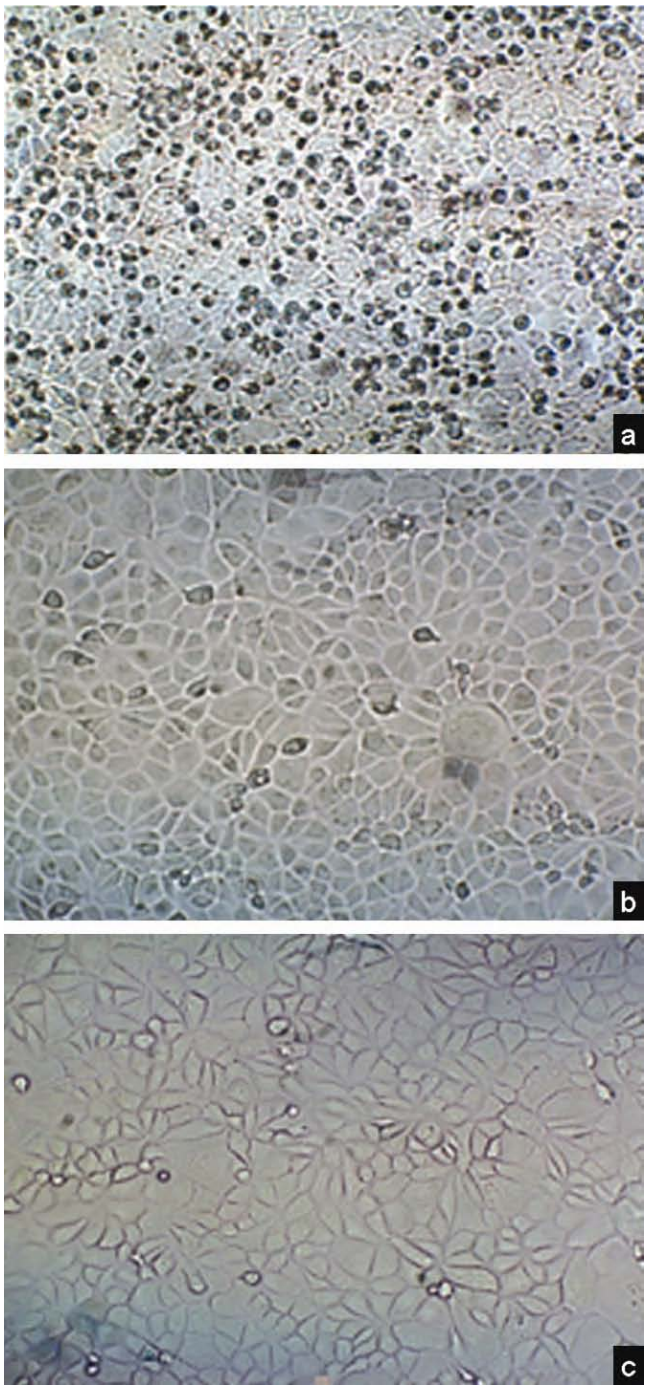

Fig. 1—CPE reduction as seen in MDCK cells after addition of cold and hot aqueous extracts of bark after 72 hr of infection with H5N1 virus. (a) Untreated MDCK monolayer after 72 hrs of infection with H5N1 virus; (b) MDCK monolayer treated with cold aqueous extract of bark of *E. jambolana* with after 72 h of infection with H5N1 virus (100% reduction); (c) MDCK monolayer treated with hot aqueous extract of bark of *E. jambolana* with after 72 h of infection with H5N1 virus (100% reduction).

since hot and cold aqueous extracts of leaves of *E. jambolana* exhibited pronounced virucidal effect (EC_{50} 15.22 and 14.82 µg/mL) but did not show HI, it can be concluded that blocking of HA may not be the primary action responsible for antiviral effect of *E. jambolana* extracts. Alteration of viral membrane or interference with the virus-cell membrane fusion can't be ruled out as has been reported for anti-influenza effect of Catechins[17] and tea extracts[28]. Nevertheless, the mechanism of this inhibition needs to be studied in more detail.

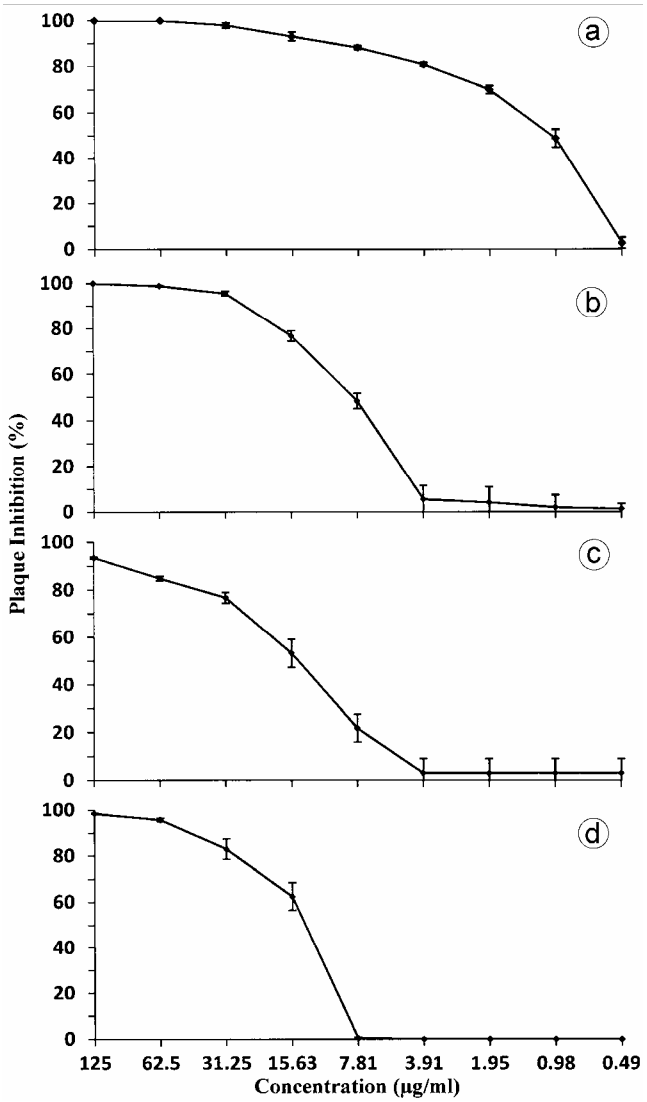

Fig. 2—Dose-response curve of extracts of *E. jambolana* (a) hot aqueous bark (b) cold aqueous bark (c) hot aqueous leaves and (d) cold aqueous leaves. A fixed amount of virus (200 pfu) was incubated with different concentrations of the crude extracts followed by estimation of virus by plaque assay. The percent inhibition of virus (pfu) in the extract-treated virus samples decreased alongwith the concentration of extract(s).

In the present study, the virus treated with hot aqueous extract of bark and cold aqueous extracts of leaves of *E. jambolana* infected only 20% of the inoculated eggs as against 100% infection in case of untreated virus control. Earlier, *in ovo* method has been used to test the antiviral activity of herbal extract on H9N2 influenza virus and expressed as percent infectivity[30]. However, in the present study, apart from percent egg infectivity, the mean HA titre of the virus in the infected eggs was also determined. The hot aqueous extract of bark and cold aqueous extracts of leaves were able to reduce the mean HA titre in the infected eggs to 2^5 (88% reduction) and $2^{7.1}$ (47% reduction), respectively from the titre of 2^8 obtained in the untreated virus control indicating a marked antiviral effect on the virus. In amantadine treated virus (drug control), only 10% of eggs got infected with reduction in mean HA titre to 2^6 (75%). The reduction in percent infectivity depends on the inactivating effect of extract on the virus, either before inoculation or in early phase of growth of virus. In contrast, the reduction in HA titre in the infected eggs indicates absence of complete inactivation of the virus and inhibition of growth of the virus during the incubation period probably due to a persistent inhibitory effect of the extract on virus replication within the egg environment. None of the eggs inoculated with virus treated with cold aqueous extract of bark and hot aqueous extract of leaves of *E. jambolana* were infected indicating the highest virucidal activity in these extracts (Table 6). These results corroborated with the results of virus yield reduction (98-99% inhibition) in MDCK cells. Moreover, 88% reduction in the mean HA titre in the infected eggs further confirmed significant virus inhibition by hot aqueous extract of bark of *E. jambolana.*

The present findings, thus, highlight the potential of crude extracts of *E. jambolana* as effective antiviral agents and provide the scientific basis for possible therapeutic use of *E. jambolana* for avian influenza virus (H5N1). Although the compounds contributing to the antiviral effects are still unclear and need to be studied further, this does not detract from the effectiveness of the studied extracts on H5N1 virus. Therapy with synergistically active antiviral compounds that have different mechanism of action may provide several advantages over single compound treatment such as greater potency, less side effects and toxicity and better clinical studies. The present findings encourage the need for clinical studies to explore the therapeutic and prophylactic

Table 4—Virucidal effect of *E. jambolana* extracts on H5N1 virus by virus yield reduction assay.

[Values are mean ± SD from 3 independent assays. Figures in parenthesis are percent reduction from virus control]

Extract	Virus yield reduction assay (pfu/mL)
Virus control	316.67±53.68
Bark (hot aqueous extract)	0.67±.1.15 (99.79)
Bark (cold aqueous extract)	1.33±2.31 (99.58)
Leaves (hot aqueous extract)	3.33±3.06 (98.95)
Leaves (cold aqueous extract)	5.33±4.16 (98.31)
Amantadine	44.33±4.04 (86)

The extracts and the control drug were tested at respective MNTC as shown in Table 1.

Table 5—Inhibitory effect of various extracts of *E. jambolana* on virus adsorption to chicken red blood cells

Extract with virucidal activity	Concentration of extract (µg/mL)
Bark, hot aqueous extract	24*
cold aqueous extract	24*
Leaves, hot aqueous extract	negative
cold aqueous extract	negative

*Minimum concentration that completely inhibited adsorption of virus

Table 6—*In ovo* virucidal activity of extracts of *E. jambolana*

Extract	Number of eggs infected (HA titre in infected eggs (log2))			Total number of eggs infected	Infection (%)	*Mean HA titre in infected eggs (log2)	Reduction in HA titre in infected eggs (%)
	Exp I	Exp II	Exp III				
Untreated virus control	3 (8, 8, 8)	3 (8, 8, 8)	4 (8, 8, 8, 8)	10	100	8.0	-
Bark (hot aqueous extract)	0	1 (5)	1 (5)	2	20	5.0	88
Bark (cold aqueous extract	0	0	0	0	0	0	0
Leaves (hot aqueous extract)	0	0	0	0	0	0	0
Leaves (cold aqueous extract)	0	1 (4)	1 (8)	2	20	7.1	47
Amantadine	0	0	1 (6)	1	10	6	75

The extracts were tested at their respective MNTCs as mentioned in Table 1.
Value in parenthesis are $\log_2$ HA titer in the infected eggs.
*Mean of HA titre of the infected eggs in Experiment I, II and III (*n*= total number of eggs infected in each case).

potential of extracts of *E. jambolana* and to extend this study to other influenza viruses for finding out treatment of H5N1 pandemic influenza.

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