ORIGINAL ARTICLE

Antiviral activity of Indian medicinal plants against pandemic 2009 Influenza (H1N1) virus

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Abstract

Introduction: Crude extracts of leaves and stems of 16 Indian medicinal plants were tested for their antiviral activity against 2009 pandemic influenza virus (H1N1) by neutralization, hemagglutination inhibition, and cytopathic effect reduction assay. **Materials and Methods:** Seventeen medicinal plants were extracted to prepare 50% ethanol and aqueous extract. These 34 extracts were tested for anti-influenza properties at their non-toxic concentrations. **Results:** On the basis of microneutralization assay, both 50% ethanolic, aqueous extracts of *Rhododendron arboreum and Pulmonaria longifolia* and 50% ethanolic extracts of *Rollinia parviflora* showed better potential (IC_{50} value of 1.93, 3.35, and 3.75 µg/ml, respectively). In cytopathic effect reduction assay, *Salix alba* aqueous and 50% ethanol extracts of *R. arboreum and Urtica dioica* (IC_{50} value of 0.980, 2.14, and 2.33 µg/ml, respectively) demonstrated anti influenza activity. The results suggest that selected plant extracts have a protective effect against pandemic H1N1 virus infection. **Conclusion:** The results indicates that the experimental plants demonstrate promising antiviral activity and could prove effective alternate against conventional allopathic drugs.

Key words: Hemagglutination, Indian medicinal plants, microneutralization, pandemic influenza virus

INTRODUCTION

Plants have been used as a source of medicine from ancient time till date all over the world. Although many plants have long been recognized and widely used in Indian traditional medicines, some are relatively unexplored and have not entered into mainstream medicines.[1] Natural products from plants can be source of innovative, safer, affordable, and powerful therapeutic agents.[2,3] For example, screening of plants as a possible source of antiviral drugs has led to the discovery of potent inhibitors of *in vitro* viral growth.[4-11] The present investigations were carried out to assess the antiviral effects of selected Indian plants used by the local people for various ailments.

In particular, the studies were undertaken keeping in view of 2009 H1N1 Swine origin influenza A virus pandemic infection that has caused more than 1.4 million infections and resulted in approximately 25,000 deaths worldwide.[2] 2009 Pandemic in human was caused due to novel highly contagious influenza (H1N1) belonging to type A influenza virus of family Orthomyxoviridae. Antiviral drugs such as oseltamivir, zanamivir, amantadine, and rimantadine have been considered critical in preparedness for H1N1 virus originated pandemic. Several at risk nations and WHO have stored strategic stockpiles of antivirals, especially oseltamivir to be used at a face of influenza pandemic. However, side effects resistance to oseltamivir in the H1N1 subtype in India and other human influenza A viruses has become a cause of worry as far as pandemic preparedness is concerned.[12-14] Therefore, the search for alternative antivirals that can effectively inhibit H1N1 or act in synergy with available antivirals is imperative. Several novel antiviral agents that may be effective against influenza virus, especially the pandemic H1N1 flu virus, are currently under development.

In India, many Indian medicinal plants and traditional prescriptions have a long history of clinical application. Indeed, they are being utilized as anti-influenza agents without major side effects. Therefore, the extracts of these plants were studied as candidate against 2009 H1N1 S-OIV (Swine origin influenza A virus; hereafter referred to as pandemic H1N1)

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that has caused more than 5 million infections with about 200,000 deaths worldwide.[14] In this field, plant-derived extracts have become focus of many studies due to their proven beneficial health effects in several disease problems. Many Indian medicinal plants and traditional prescriptions have a long history of clinical application with some of them being utilized against flu-like illnesses without major side effects. Therefore, the extracts of selected plants were studied as candidate anti-influenza virus agents, especially the pandemic H1N1 virus. These candidates may be useful as prophylactic or therapeutic agents in the future.

In this study, 16 Indian medicinal plants were screened for initial anti H1N1 properties. Among them, three plants were selected for further the study: *Rhododendron arboreum*, *Pinus longifolia*, and *Rhus parviflora*.

Medicinal uses of Selected Indian Medicinal Plants

R. arboretum Sm. (Ericaceae) is a tree species endemic to the southern Western Ghats of peninsular India. Its dried flowers are used in checking diarrhea and blood dysentery. In addition, flavonoids such as hyperoside (3-D-galactoside of quercetin), ursolic acid, epifriedelinol; quercetin-3-rhamnoside quercetin, rutin, and coumaric acid have been isolated from different parts of *R. arboretum*.[15,16]

Rhus parviflora Roxb. (*Anacardiaceae*) distributed in Nepal, northern India, Bhutan and Sri Lanka, has been used in neurological disorders including anxiety, insomnia, epilepsy, and rheumatoid arthritis. Phytochemically, Gallic acid, some flavones, namely, myricetin, quercetin, myricitrin, quercitrin, kaempferol, and glycosides (isorhmnetin-3-α-Larabinoside) have been isolated from this plant.[17]

P. longifolia Sarg. is a species of several evergreen trees belonging to the Pinaceae and is native to the Himalayas and distributed throughout Pakistan, India, Nepal, and Bhutan. Its anti-fungal and anti-bacterial activity is already known[18] Studies conducted using *Pinus parviflora* pine cone extract have shown effective suppression of the growth of influenza virus by inhibiting viral protein synthesis in infected cells and virion-associated RNA-dependent RNA polymerase activity.[19] Flavonoids like kaempferol have been shown to have neuraminidase inhibitory activities against two influenza viruses, H1N1 and H9N2.[20]

MATERIALS AND METHODS

Collection Plant and Preparation of Extracts

Plant material was collected from Lucknow, India, during June–November 2018. Their specimens were submitted in the form of herbarium sheets in National Botanical Research Institute, Lucknow. Authenticated plant material was shade dried leaves and stems were grinded and strained through 30 meshes (0.5 mm). The voucher specimen has been submitted to Herbarium of National Botanical Research Institute, Lucknow, India. The finely grinded plant materials (500 g) were extracted with MilliQ water (1.5 L × 3) at 60–75°C for 6–8 h to obtain aqueous extracts of leaves and stems. These extracts were filtered and concentrated under reduced pressure to afford aqueous extracts.

Cell Lines and Virus

Madin-Darby Canine Kidney (MDCK) cell line (CCL-34) was obtained from American Type Culture Collection, Manassas, VA, USA and cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich Inc., St. Louis, MO, USA). Medium was supplemented with 10% fetal bovine serum and an antibiotic-antimycotic cocktail (Penicillin [100 units/ml], Streptomycin [100 µg/ml], and Amphotericin B [0.25 mg/ml]; Biological Industries, Kibbutz beit, Haemek, Israel). MDCK cells were cultured at 37°C under humidified conditions with 5% CO_2. Pandemic H1N1 NYMCX-179A (A/California/07/2009: Influenza virus infectious NYMCX-179A, NIBSC Code 09/124) received from NIBSC, UK and passaged in MDCK cells in presence of TPCK-trypsin (2 µg/ml; Sigma Aldrich Inc.) which was kindly provided by Serum Institute of India, Limited, Pune India. Titer of the virus stock was calculated using Reed and Muench method.[21]

Cytotoxicity Assay

Cytotoxicity of the plant extracts was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich Inc.) as described previously.[22] Briefly, MDCK cells (1.5×10^3/well/100 µL) were seeded in a 96-well culture plate (Greiner Bio-One, GmbH, Frickenhausen, Germany) and grown overnight at 37°C in a humidified atmosphere of 5% CO_2. Next day, culture medium with increasing concentrations of various extracts (62.5–500 µg/ml) was added in duplicate and, further, incubated for 48 h. The solvent used to prepare extracts was included as negative control. After incubation, 20 µL of MTT reagent (5 mg/mL) was added per well and incubated at 37°C for 3 h followed by addition of MTT solvent (100 µL/well; 20% [w/v] sodium dodecyl sulfate [SDS] and 50% (v/v) N, N-dimethyl formamide (DMF) in 50 mM PBS). The absorbance (OD) was read at 570 nm with reference filter at 690 nm. Cell viability was calculated using the equation,

% Viability = [(OD extract treated cultures)/(OD solvent treated cultures)]×100

Microneutralization assay

MDCK cells (1.5×10^4/well) were seeded in 96-well cell culture plate (Greiner Bio-one) and incubated overnight

in humidified atmosphere and 5% CO_2 at 37°C. After 24 h, cells were incubated with 100 µl of serial dilution of plant extract (50, 25, 12.5, and 6.25 µg/ml) in serum free DMEM medium in duplicate for 1 h. Cells were, further, infected with 100 µl of 30 $TCID_{50}$ units of the virus and incubated for 24 h at 32°C under humidified atmosphere and 5% CO_2. After incubation, cells were washed twice with 100 µl of PBS and fixed with 80% acetone. Fixed cells were probed with 1:4000 dilution of anti-influenza A-nucleoprotein antibody (Millipore, Billerica, MA, USA). After 1 h of incubation, cells were washed thrice with 50 mM PBS pH 7.0 supplemented with 0.1% Tween 20 (PBST). The plates were further probed with 1:10000 dilution of HRP conjugated goat-anti-mouse antibody (Pierce Biotechnology Inc., Rockford, IL, USA) for 1 h at 37°C. Cells were washed 3 times with PBST and developed using o-phenylene-diamine (0.5 mg/ml; Sigma Aldrich Inc.) and hydrogen peroxide (0.06%; Merck, Mumbai, India) in citrate-phosphate buffer (pH 5). Optical density was measured at 490 nm with a reference wavelength of 630 nm after adding 50 µl 5N H_2SO_4 as a stop solution. The 50% inhibitory concentration (IC_{50}) of the plant extract was calculated using the non-linear regression program of GraphPad Prism software (San Diego, California).

Anti-influenza Activity using Cytopathic Effect Inhibition Assay

MDCK cells (1.5×10^3 cells/well) were seeded in 96-well tissue culture plates and incubated at 37°C for 24 h in a humidified 5% CO_2 atmosphere. The cell cultures were preincubated with serial dilutions of plant extracts in medium (50, 25, 12.5, and 6.25 µg/ml) at final concentration of 100 µl in duplicate for 1 h at 37°C. Cells were, then, infected using 30 $TCID_{50}$ of influenza virus followed by the incubation for 72 h at 32°C. MTT colorimetric assay[21] was performed to test antiviral activity. Twenty microliters of MTT (5 mg/ml in 50 mM PBS pH 7) were added to each well and incubated for 4 h at 37°C. Optical density was measured at 540 nm after dissolving the formazan crystals with 100 µl of 20% SDS, 50% DMF.[23] The anti-influenza virus activities of the plant extracts were evaluated as % cell survival relative to cells in negative solvent control well (100% survival).

Hemagglutination Inhibition (HI) Assay

In addition, the plant extracts were also evaluated for their ability to inhibit the binding of H1N1 virus to the sialic acid residues present on red blood cells, using HI assay.[22] The HI assay was carried out in V-bottom 96-well plate (Greiner Bio-one, GmbH, Germany) using 4HA units of the pandemic H1N1 virus and 0.5% guinea pig RBCs. The plant extracts were used at a concentration of 12.5 µg/ml and treated with neuraminidase (Denka Seiken Ltd., Tokyo, Japan) before the assay, to prevent non-specific activity.

RESULTS

Cytotoxic Effects of the Plant Extracts on MDCK Cells

In this study, the aqueous extracts from different parts of 16 medicinal plants belonging to different families were screened for their antiviral activity against pandemic H1N1 virus [Table 1]. As a prerequisite for antiviral activity, the cytotoxicity of all the extracts was evaluated against virus-host cell (MDCK) using MTT-based cytotoxicity assay. The results are summarized in Table 2.

No cytotoxicity was observed with aqueous extract prepared from most of the test plant extracts and was found to be non-toxic on MDCK cell lines up to 60 µg/ml concentration [Table 2]. However, extracts prepared from *Salix alba, Terminalia chebula,* and *Cannabis indica* demonstrated moderate cytotoxicity with CC_{50} values (the concentration that causes the reduction of viable cells by 50%) of 229.7, 154.1, and 179.0 µg/ml, respectively. Highest toxicity showed by aqueous extract prepared from *Rollinia parviflora* (CC_{50} value 58.66 µg/ml).

Anti-influenza Virus Activity using Microneutralization Assay

The microneutralization assay is a highly sensitive and specific assay for detecting virus specific neutralizing antibodies to influenza viruses in human and animal sera. In our experiments, most promising results were obtained from aqueous extract prepared from aqueous extract of *R. arboreum* and *Pulmonaria longifolia* (IC_{50} values 1.93 and 3.35 µg/ml, respectively, Figure 1). Whereas *R. parviflora* and *Urtica dioica* showed moderate profile with IC_{50} values of 3.75 and 3.91 µg/ml, respectively.

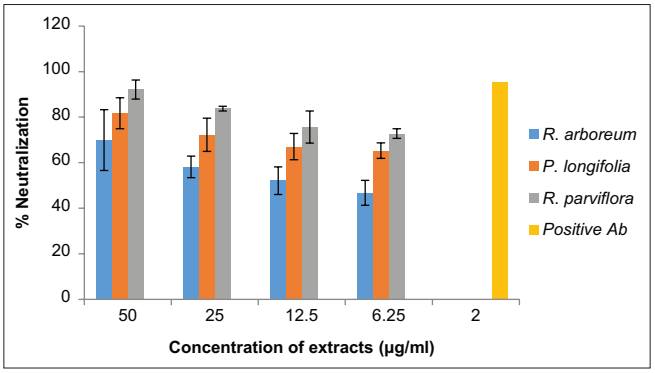

Figure 1: *In vitro* microneutralization assay using pandemic H1N1 virus: The aqueous extracts of *R. arboretum, P. longifolia* and *R. parviflora* were tested at varying concentrations (50 to 6.25 µg/ml) against 30$TCID_{50}$ dose of pandemic H1N1 virus. Pandemic H1N1 neutralizing MAb MA2077 at 2 µg/ml was used as internal positive control. Values are expressed as mean ± SE of 2 different experiments performed in duplicates.

Table 1: Indian medicinal plants used in the present study.

S. No.	Plant names	Family	Part used	Major traditional use (s)
1.	*Rhododendron arboreum* Sm.	Ericaceae	Leaves	Diarrhoea and blood dysentery[24]
2.	*Argemone mexicana* L.	Papaveraceae	Roots	Skin diseases, leprosy, fevers[25]
3.	*Acacia catechu* Willd.	Leguminosae	Stem bark	Bark is antioxidant, anti-inflammatory, anti-bacterial and antifungal in nature[26]
4.	*Citrus karna* Raf.	Rutaceae	Fruits	Strong antioxidant, anti-atherogenic, anti-viral, anti-mutagenic, antiulcer and antitumor effects[27]
5.	*Andrographis paniculata* Nees.	Acanthaceae	Whole plant	Hepatoprotective, antimalarial, antihypertensive, antipyretic[28]
6.	*Tinospora cordifolia* Willd.	Menispermaceae	Whole plant	Anti-tumour, neuroprotective activities[29]
7.	*Lagerstroemia speciosa* L.	Lythraceae	Leaves	Treatment of diabetes, obesity and kidney related diseases[30]
8.	*Urtica dioica* L.	Urticaceae	Whole plant	Treat rheumatic pain, colds and cough[31]
9.	*Salvadora persica* Wall.	Salvadoraceae	Stem	in the prevention of tooth decay[32]
10.	*Pinus longifolia* Roxb.	Pinaceae	Leaves	Wood act as antiseptic, stimulant and carminative[18]
11.	*Rhus parviflora* Roxb.	Anacardiaceae	Leaves	In neurological disorders, insomnia, epilepsy and rheumatoid arthritis[17]
12.	*Rubia cordifolia* L.	Rubiaceae	Leaves	Treat amenorrhoea, diarrhoea, renal calculi, jaundice and paralysis[33]
13.	*Salix alba* L.	Salicaceae	Whole plant	anti-inflammatory property[34]
14.	*Terminalia chebula* Retz.	Combretaceae	Leaves	In treatment of asthma, sore throat, vomiting, bladder diseases[35]
15.	*Alstonia scholaris* L.	Apocynaceae	Leaves	Treatment of skin, liver disease, dysentery, ulcers, tumors, malaria and fever[36]
16.	*Canna indica* L.	Cannaceae	Fruits	Antinociceptive and anthelmintic properties[37]

Anti-influenza Activity using Cytopathtic Effect Inhibition Assay

Antiviral activity against pandemic H1N1 influenza virus using cytopathic effect inhibition assay was carried out at their non-cytotoxic concentrations using MTT reagent. However, aqueous extract prepared from *S. alba* proved most effective with IC_{50} value of 0.98 µg/ml followed by *R. arboreum* extract (IC_{50} 2.14 µg/ml) [Figure 2]. *Andrographis paniculata* and *U. dioica* demonstrated moderate profile with IC_{50} value of 3.98 and 2.33 µg/ml, respectively. Activity profile in terms of IC_{50} values of other plant extracts ranging from 5.58 ~ 22.67 µg/ml.

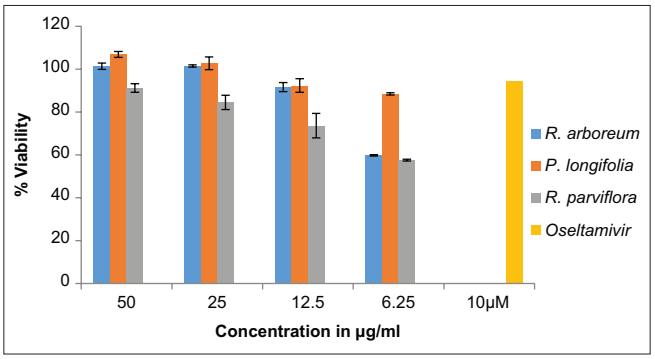

Figure 2: Cytopathic effect inhibition assay using pandemic H1N1 virus: The aqueous extracts of *R. arboreum, P. longifolia* and *R. parviflora* were tested at varying concentrations (50 to 6.25 µg/ml) against 30$TCID_{50}$ dose of pandemic H1N1 virus in cytopathic effect inhibition assay. Neuraminidase inhibitor, Oseltamivir, was used as internal positive control at 10 µM concentration. Values are expressed as mean ± SE of 2 different experiments performed in duplicates.

HI Assay

The plant extracts were also evaluated for their ability to inhibit sialic acid binding, and thus, neutralization of the influenza virus, using HI assay, was performed [Figure 3]. The HI assay showed that two of the extracts, *R. arboreum* and *R. parviflora,* showed HI activity against the pandemic H1N1 virus, whereas *P. longifolia* extract failed to show any HI activity [Table 2].

The IC_{50} values of the plant extracts differ in the microneutralization and cytopathic effect inhibition assays. The reason may be

Table 2: Cytotoxic effects and anti-pandemic H1N1 activities of Indian medicinal plant extracts.

S.No.	Plant species	Cytotoxicity† (CC_{50} µg/ml)	Antiviral activity (concentration in µg/ml)		
			Neutralization assay‡ (IC_{50})	Cytopathic inhibition assay§ (IC_{50})	Hemagglutination¶ inhibition
1	*Rhododendron arboreum* Sm.	>500	1.93	2.14	+
2	*Argemone mexicana* L.	>500	14.05	5.58	-
3	*Acacia catechu* Willd.	>500	26.98	15.41	-
4	*Citrus karna* Raf.	>500	10.72	7.88	-
5	*Andrographis paniculata* Nees.	>500	>50	3.98	-
6	*Tinospora cordifolia* Willd.	>500	7.04	9.53	-
7	*Lagerstroemia speciosa* L.	>500	8.98	11.89	-
8	*Urtica dioica* L.	>500	3.91	2.33	-
9	*Salvadora persica* Wall.	>500	-	22.67	-
10	*Pinus longifolia* Roxb.	>500	3.35	12.79	-
11	*Rhus parviflora* Roxb.	58.66	3.75	13.49	+
12	*Rubia cordifolia* L.	>500	-	9.99	-
13	*Salix alba* L.	299.7	10.82	0.98	-
14	*Piper betle* L.	>500	4.73	5.90	-
15	*Terminalia chebula* Retz.	154.1	61.80	6.73	+
16	*Canna indica* L.	179.0	87.78	11.51	-

†The cytotoxicity of plant extracts was tested on MDCK cell line using MTT reagent. CC50 value represents the cytotoxic concentration of the extracts that caused the reduction of viable cells by 50%. All data presented are averages of duplicate experiments. ‡The activity of plant extracts was also evaluated in an *in vitro* microneutralization assay using 30TCID50 dose of pandemic H1N1 virus. IC_{50} value represents 50% inhibitory concentration of the extracts that neutralized 50% of the virus as compared to virus control well. §The cytopathic inhibition activity of plant extracts was evaluated against $30TCID_{50}$ dose of pandemic H1N1 virus on MDCK cell line using MTT colorimetric assay. ¶The HI assay was performed using 4HA units of pandemic H1N1 virus and 0.5% guinea pig RBCs.

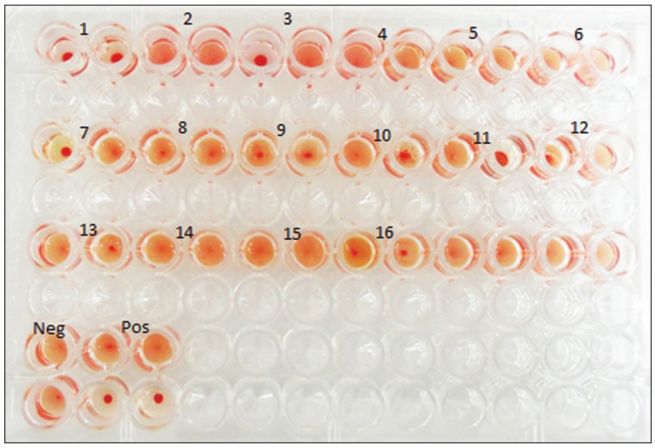

Figure 3: Hemagglutination inhibition (HI) assay using pandemic H1N1 virus: 16 plant extracts were treated with 4HA units of the pandemic H1N1 virus and 0.5% guinea pig RBCs. Positive extracts The HI assay showed that the extracts prepared from leaves of *R. arboreum* and *R. parviflora* showed significant clumping (positive HI activity) against the pandemic H1N1 virus; whereas *P. longifolia* extract failed to show any HI activity. Test sample was analyze in duplicates and such 3 independent experiments were performed for validity testing.

speculated as some of the plant extracts are able to act at the entry level of the virus as evident from the microneutralization assay, whereas those extracts showing cytopathic effect inhibition might be acting at the later steps of viral replication. Moreover, it is possible that plant extracts showing HI activity are inhibiting sialic acid binding steps of the influenza virus. However, these studies would require further analysis.

Nonetheless, our results indicate that several plants used in Indian traditional medicine could be lead to potential antiviral drugs, which may possibly provide molecules with drug-like properties and with incredible structural diversity. The phytochemical characterization of the extracts, the identification of the responsible bioactive compounds, and elucidation of the mode of action and quality standards are necessary. The phytochemical knowledge about these plants regarding their anti-pandemic H1N1 virus activity is not reported so far and their active principles were still under investigation.

Influenza virus infects the mucous membranes of the upper respiratory tract and occasionally invades the lungs, as a consequence, secondary bacterial infection may occur in susceptible individuals such as infants and the elderly.[38,39] The previous studies indicate that several plants used in Indian traditional medicine could be the lead to potential antiviral drugs, which possibly provide molecules with drug-like properties and with incredible structural diversity.[40] Besides, the results in this study are useful for rationalizing the use of medicinal plants in primary healthcare in India. The phytochemical

characterization of the extracts, the identification of the responsible bioactive compounds, and elucidation of the mode of action and quality standards are necessary.

Among the test extracts aqueous extract prepared from *R. arboreum, S. alba,* and *A. scholaris* were found most active. The extracts that exhibited only medium and low activity could also be the source of potential antiviral drugs, because the bioactive compounds may be present in too low concentrations to show effective antiviral activity at non-toxic concentration. Further, fractionation and separation of extract(s) may reveal potent antiviral activity.

DISCUSSION

The results of this work justify the potential of some of the investigated plants for the production of bioactive compounds. The phytochemical knowledge about these plants about anti H1N1 pandemic flu virus is not reported so far and their active principles were still under investigation.

CONCLUSION

Based on the above studies it was clear that extracts from selected medicinal plants with variable plant families demonstrates changes in antiviral activity against H1N1 pandemic flu. The metabolite variation and antiviral activity of the plant extracts suggest that it can be used as a source for new therapeutic compound development against H1N since this viral disease was spread worldwide.

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