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RESEARCH ARTICLE

ASSSESSMENT OF LEAF EXTRACTS OF INDIAN MEDICINAL PLANTS FOR ANTI-INFLUENZA VIRUS ACTIVITY

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ABSTRACT

Virus is responsible for causing large number of morbidities and mortalities across the globe. Although there are effective anti-virals against the virus, the high rate mutation leads to constant changes in the viral protein structures. This may lead to development of resistance against the existing drugs. The present study was undertaken to assess the in vitro anti-influenza virus activity of *Mangiferaindica* (Mango), *Aegle marmelos* (Bael) and *Ricinus communis* (Eranda). Methanol extracts of the leaves from these plants were prepared using the Soxhlets apparatus. Total proteins were extracted using commercially available kit. Experiments were performed using Influenza Virus A/H1N1/PUNE/2009; standard virus obtained from National Institute of Virology, Pune, India. Pre and post exposure assays were carried out on Madin Darby Canine Kidney (MDCK) Cells. Reduction of Hemagglutination Titre was used as a marker for anti-viral activity. The methanol extract of Mango and protein extract of Bael have potential anti-influenza virus activity, as exhibited by hemagglutination inhibition. Even at 100 g/mL the HA titre of 1:64 of the Virus control was reduced to 1:8 in case of methanol extract of mango. Protein extract of Bael exhibited a 1:8 titre at 1000 g/mL. In the event of constant mutations in the influenza virus novel antivirals will be useful in case of future epidemics.

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INTRODUCTION

Influenza has been known to cause high morbidity and mortality since a long time. The Spanish flu of 1918 killed around 50 million people(Taubenberger& Morens, 2006). The infection is caused by a diverse strain of the influenza virus. In any given year, some strains of influenza virus can cause seasonal disease while others may cause epidemics, with a potential to cause pandemics. During the past decade the world has witnessed the global occurrence of seasonal influenza, the manifestation of avian influenza due to the influenza A (H5N1) virus with a high mortality rate in many countries, and the wave of a pandemic in 2009 due to a novel strain of influenza (pH1N1). While seasonal influenza occurs every year, epidemics may occur after several years and pandemics strike once in several decades(Kamps, Hoffmann, & Preiser, 2006). The 2009 pandemic and the emergence of influenza viruses underline themassivetrials to global control of the disease by vaccination and currently available antivirals. Although Antiviral therapy and prophylactic vaccination are available controlling an influenza virus infection, development of anti-viral drug resistance and the challenge to make vaccine appropriate to counter the seasonal variations before a new virus strain emerges are major challenges. Hence there is an urgent need to screen for novel anti-viral against influenza viruses *Mangiferaindica* (Mango), *Aegle marmelos* (Bael) and *Ricinus communis* (Eranda) have traditionally been used in Ayurveda and folk-lore medicine in India as anti-inflammatory agents and as anti-microbials.Especially mango and bael has been used in the treatment of respiratory disorders(Abubakar, 2009; Reddy, Reddy, & Trimurthulu, 2006).Therefore, the present study was conducted to assess the anti-viral effect of methanol and protein extracts from the leaves of *Mangiferaindica* (Mango), *Aegle marmelos* (Bael) and *Ricinus communis* (Eranda) against the influenza virus in vitro.

MATERIALS AND METHODS

All experiments were done after obtaining due ethics committee clearance of the Institutional Ethics Committee of the Haffkine Institute, vide letter no. HITRT/IEC/13/2011.

Collection and Plant Identification: The plant was collected from Haffkine Institute campus in the monsoon season and a specimen in the form of a twig was sent to the 'Botanical Survey of India', Pune, for authentication.

Processing of the plants: The leaves of *Aegle marmelos* (Common Name: Bael)*, Ricinus communis* (Common Name: Eranda) and *Mangiferaindica* (Common Name: Mango) were collected and washed thoroughly with water. They were then kept for shade drying. The dried leaves were ground to fine powder and were used in the extraction. Organic methanol extracts were made using Soxhlet method. Total proteins were extracted using commercially available P-PER plant protein extraction kit (M/s. Thermo Fisher Scientific) ("P-PER ® Plant Protein Extraction Kit," n.d.). The samples were labelled as AM, RC and MI : Methanol extract of Bael, Eranda and Mango respectively and MP, RP and MIP: Protein extract of Bael, Eranda and Mango respectively.

Phytochemical Profiling: Phytochemical assessment and Fingerprinting of the methanol extracts were performed by HPTLC method. High Performance Thin Layer Chromatography (HPTLC) is a sophisticated and automated form of Thin layer chromatography (TLC). Fingerprinting of methanol extracts of the leaves were carried out to identify the components present in it. The above samples were dissolved in their methanol solvents to give a final concentration of 1 mg/ mL. The samples were sonicated for 10 minutes for obtaining a homogenous mixture which was then loaded into the syringe. The samples were screened for Flavanoid, Phenolics, Tannins, Glycosides, Alkaloids and Sterols. CAMAG Linomat 5 system which is a semi-automated system was used for the loading of the sample and standards. 10µL of samples and standards was loaded onto a TLC silica gel aluminium sheet (20 mm x 10 mm) through a syringe. The mobile phase and derivatization were as mentioned in Table 1.

Anti-Viral Assay

Preparation and Titration of Influenza Virus(Patil et al., 2013): Experiments were performed using Influenza Virus A/H1N1/PUNE/2009; standard virus obtained from National Institute of Virology, Pune, India. Influenza virus stocks were grown in cell culture using Madin Darby canine kidney (MDCK) cells and titre of infectious virus were measured by median tissue culture infective dose ($TCID_{50}$) titration. MDCK cells for viral titration were grown in complete MEM. Confluent MDCK cell monolayer in 96-well tissue culture plates were washed once with serum-free-MEM before use. Serial 10-fold dilutions of virus in serum-free-MEM containing 0.3 % BSA and 2 µg/mL l-(toslyamido 2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin and Nystatin (50 U/mL) were incubated in replicate wells (200 µL/well) for 4 days at 37 °C with 5 % CO_2. Wells positive for virus growth were identified by staining the cells with 0.1 % trypan blue and TCID50 titre were calculated by the method of Reed and Muench

Study on inhibition of Influenza hemagglutinin protein (Patil et al., 2013): Dried plant extracts were dissolved in DMEM without phenol red and required dilutions were prepared. Confluent MDCK cell cultures were treated with different dilutions of methanol and aqueous extract in three sets of experiments as follows:

Pre-exposure Assay: Virus stock of $10^{2.5}$ $TCID_{50}$ (64 HA titre) was exposed to effective minimal cytotoxic concentrations of plant extracts for 1 h at 37 °C. Then 100 µL of the mixture was added to the cells cultured fluently in 96-well flat-bottom microtitre plate. Following 1 h incubation at 37°C, the supernatants were removed and the cells were washed with phosphate buffered saline (PBS). Then 100 µL of the TPCK-containing medium was added to each well.

Post- exposure Assay: Virus stock of $10^{2.5}$ $TCID_{50}$ (64 HA titre) (100 µL/well) was added to each well. After 1 h incubation at 37 °C the unabsorbed viruses were removed and cells were washed and 100 µL of plant extracts diluted with TPCK-containing medium was added to each well. All the plates including MDCK cells (Cell control) and influenza virus inoculated MDCK cells were incubated at 37°C in CO_2 incubator for 48 h and the virus titration was carried out by Hemagglutination assay.

Hemagglutination assay: In order to assess the presence of the virus in cell culture, serial dilutions of the cell culture media (50 µL/well) were added in 96-well U-shaped microtitre plate. Human O red blood cells (0.75 %) were added to each well (50 µL/well). Following incubation at least for 1 h at room temperature, the virus titre of each well was recorded. A diffused sheet of agglutinated erythrocytes covering the bottom was regarded as 100 % hemagglutination, while a well circumscribed button of non-agglutinated erythrocytes was regarded as non-hemagglutination. The HA titre of the supernatant was defined as the reciprocal of the highest dilution showing complete hemagglutination. Complete settling of the RBCs demonstrated absence of the virus while hemagglutination indicated the presence of the virus.

RESULTS

Phytochemical Profiling

Results for Phytochemical profiling are compiled in Table 2.

In vitro cytotoxicity assay: The *in vitro* cytotoxicity of medicinal plant extracts (Bael, Eranda and Mango) were evaluated by MTT assay and the median cytotoxicity (CC50) of Methanol extract of Bael (AM), Eranda (RC) and Mango (MI) were found to be 11.58 mg/mL, 11.63 mg/mL and 7.063 mg/mL whereas Protein extract of Bael (MP), Eranda (RP) and Mango (MIP) were found to be 25.68 mg/mL, 0.1362 mg/mL and 2.075 mg/mL respectively Table 3.

Hemagglutination Inhibition assay: In the pre-exposure experiments, anti-influenza virus activity was seen with the methanol extract of bael (AM) exhibiting a 4-fold reduction in virus titre (1:16) at 1000 µg/mL methanol extract of mango (MI) exhibiting a 2-fold reduction in virus titre (1:32) at 1 µg/mL concentration. In the post-exposure experiments anti-influenza virus activity was seen with the methanol extract of mango (MI) and protein extract of bael (MP) exhibiting a 8-fold reduction in virus titre (1:8) at 100 µg/mL and 1000 µg/mL respectively, methanol extract of bael (AM) and eranda (RC) exhibited a 2-fold reduction in virus titre (1:32) at 1000 µg/mL concentration.Table 4 & 5, Figure 2 & 3.

Table 1. Mobile phase and derivatizing agents of specific phytochemicals

Phytochemical	Stationary Phase	Mobile Phase	Derivatizing agent
Alkaloid	Silica F_{254}	Ethyl acetate: methanol: gacial acetic acid (6.8:3.0:0.2)	Dragendroff reagent
Falvanoid	Silica F_{254}	Ethyl acetate: formic acid: gacial acetic acid: water (10:0.5:0.5:1.3)	Anisaldehydesulphuric acid
Phenolics	Silica F_{254}	chloroform: methanol (9.5:0.5)	Anisaldehydesulphuric acid
Sterols	Silica F_{254}	Chloroform: Ethyl acetate (4:6)	Acetic anhydride: Sulphuric acid in ethanol
Tannins	Silica F_{254}	Toluene-ethyl acetate-formic acid-methanol (3:3:0.8:0.2)	Ferric Chloride
Glycosides	Silica F_{254}	Ethylacetate-Methanol (1.3:0.5)	Kedde Reagent

Table 2. Phytochemical profiles of the extracts used in the study. ALK-Alkaloids, Flav-Flavanoids, TAN-Tannins, GLYCO-Glycosides, Pheno-Phenolics, STER-Sterols, TERP-Terpenoids

SAMPLE	ALK	FLAV	TAN	GLYCO	PHENO	STER	TERP
AM	++	+++	+	-	++++	+	-
RC	++	++	+	+	++	++++	+++
MI	+	+	++	++++	+++	++	-

Table 3. Median Cytotoxic Concentrations of the samples on MDCK cell line

MEDIAN CYTOTOXIC CONCENTRATION (mg/mL)					
AM	RC	MI	MIP	MP	RP
11.58	11.63	7.063	2.075	25.68	0.1362

Table 4. Hemagglutination (HA) Inhibition activity of the samples at various concentrations (1 µg/mL-1000 µg/mL) in pre-exposure experiments. Values are HA units exhibited by the culture supernatants when virus was pre-incubated with extracts and then exposed to MDCK cells. Virus Control exhibited an HA titre of 64

SAMPLES	HA UNITS AT VARIOUS CONCENTRATION (µg/mL)			
	1000	100	10	1
AM	16	32	64	64
RC	32	64	64	64
MI	32	32	32	64
MIP	32	32	64	64
MP	32	64	64	64
RP	64	64	64	64

Table 5. Hemagglutination (HA) Inhibition activity of the samples at various concentrations (1 µg/mL-1000 µg/mL) in post-exposure experiments. Values are HA units exhibited by the culture supernatants when virus was incubated on cells for 1 hour and then the samples were added to MDCK cells. Virus Control exhibited an HA titre of 64

SAMPLES	HA UNITS AT VARIOUS CONCENTRATION (µg/mL)			
	1000	100	10	1
AM	16	32	64	64
RC	16	32	64	64
MI	8	8	32	64
MIP	32	32	64	64
MP	8	16	16	32
RP	64	64	64	64

DISCUSSION

Of the 122 Viral families classified by the ICTV (2016), over 200 members from 22 families are known to cause infections in humans. However as of 2016, about 80 antiviral drugs have been approved to treat diseases caused by only 9 human viruses (HIV, HBV, HCV, HCMV, HSV, HPV, HRSV, VZV, and Influenza virus) encompassing 7 families(De Clercq & Li, 2016). Since accurate diagnosis of most viral infections – especially in resource limited settings – itself is a daunting task, successful and specific management of these diseases is a major challenge for modern bio-medical scientists. Also, given the fact that most viruses adapt to these anti-viral stresses and develop drug resistance, makes the process of treatment with anti-virals as 'walking a tight rope' like. The Human Immunodeficiency Virus and the Influenza Viruses are particularly notorious in this matter. HIV has around 41 approved drugs (Cihlar & Fordyce, 2016) targeted against 5 different proteins (Protease, RT, Integrase, gp41, CCR5).

On the other hand, most circulating strains of the influenza viruses are sensitive to only 4 drugs(Ison, 2011), targeted against 1 protein (Neuraminidase). The plethora of knowledge available in traditional and folklore medicines across the world may be used as starting points for screening and identification of potential anti-viral agents, especially against those that do not have an anti-viral. Hence taking a cue from Ayurveda, this study focussed on assessing the potential anti-viral activities of three important Indian plants against the influenza virus. Leaves of Mango (*Mangiferaindica*), Bael (*Aegle marmelos*) and Eranda (*Ricinus commuis*) were used in the study. Methanol extracts of the leaves were prepared using Soxhlet's extraction protocol and proteins were extracted using the P-PER plant protein extraction kit. Phytochemical profiling of the organic extracts was done using biochemical methods and confirmed by HPTLC. Methanol extracts of *M. indica* (MI) exhibited the presence of Glycosides and Phenolics, whereas extracts of *A. marmelos* (AM) exhibited the presence of

Flavonoids and Phenolics as the most abundance constituents. Terpenoids and Sterols were most abundant in the methanol extracts of *R.communis* (RC). Anti-viral effects were also carried out on the Influenza Virus. H3N2 strain of the influenza virus was cultured on MDCK cells. Methanol and Protein Extracts were added simultaneously and after infection of the cells. Potential anti-viral activity was assessed using the Hemagglutination assay. The methanol extract of Mango and protein extract of Bael have potential anti-influenza virus activity, as exhibited by hemagglutination inhibition.

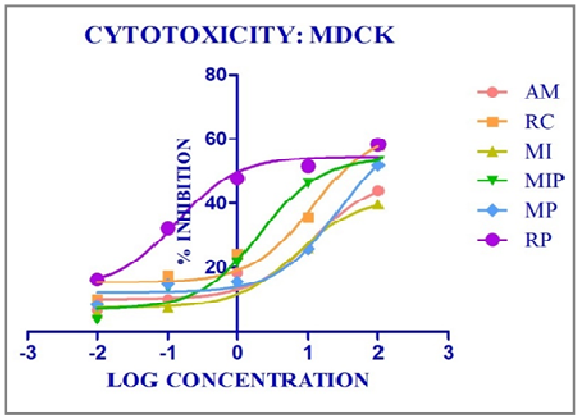

Figure 1. Graph depicting Cytotoxicity assay

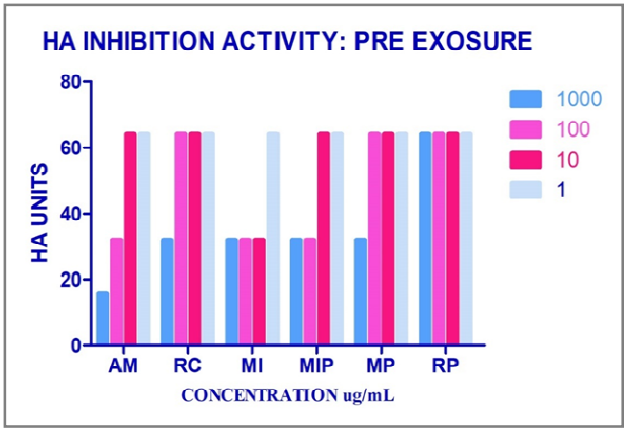

Figure 5. Graph of HA inhibition activity assay. Values on Y axis are HA units exhibited by the culture supernatants when virus was pre-incubated with extracts and then exposed to MDCK cells. Virus Control exhibited an HA titre of 64

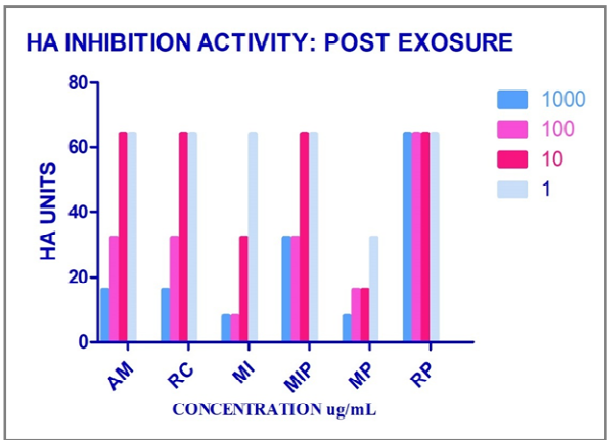

Figure 3. Graph of HA inhibition activity assay. Values on Y axis are HA units exhibited by the culture supernatants when virus was incubated on cells for 1 hour and then the samples were added to MDCK cells. Virus Control exhibited an HA titre of 64

Even at 100 µg/mL the HA titre if 1:64 of the Virus control was reduced to 1:8 in case of MI. MP exhibited a 1:8 titre at 1000 µg/mL. Many phytochemicals have been reported to have anti-viral activities. Mangiferin, a glycoside and the major constituent of alcoholic extracts from mango leaves has been shown to inhibit HSV-1 and HSV-2 in cell cultures(Zhu, Song, Huang, Wu, & Yu, 1993). It is also known to antagonize cytopathic effects of HIV in cell culture(Guha, Ghosal, & Chattopadhyay, 1996). Similarly, many active microbial and bioactive compounds have been reported from Bael. Amongst these, Marmelide has exhibited anti-viral activities against the Ranikhet Virus (New Castle Disease Virus), a paramyxovirus(Gupta, 2015) and the Human Coxsackie Virus B1-B6(Badam, Bedekar, Sonawane, & Joshi, 2002). Much work on the anti-viral activities from plant sources are limited to the secondary metabolites of plants. However, there is a small body of work which draws our attention to the potential anti-viral activities of proteins derived from plant sources. The Momordica Anti-HIV Protein 30 (MAP-30) has shown potent anti-HIV activity (Lee-Huang, Huang, Bourinbaiar, Chen, & Kung, 1995). Similarly, Gelonium Anti-HIV Protein 31 (GAP-31) isolated from *Geloniummultiflorum* has been shown to exhibit anti-HIV activity (Lee-Huang et al., 1995).

GAP-31 has been found to inhibit HIV entry and viral genome integration. It is also known to have inhibitory activities against Herpes Simplex Virus (HSV) and Human Herpes Virus 8 (HHV-8) (Bourinbaiar& Lee-Huang, 1996). The mechanisms of action of three important plant proteins – Ricin, isolated from *R. communis*, Pokeweed Antiviral Protein (PAP), isolated from *Phytolaccaamericana* and Luffa Ribosomal Inhibitory Protein (LRIP-I), isolated from *Luffa cylindriaroem* – have been elucidated and host Ribosomal Inhibition has been seen as the active principle behind their anti-viral properties(Di & Tumer, 2015; Kishida, Masuho, & Hara, 1983; Montanaro, Sperti, & Stirpe, 1973). Apart from these, gp22 and gp35 – two proteins isolated from the Tobacco plant infected with Tobacco Mosaic Virus (TMV) have exhibited anti-viral activities against the TMV(Edelbaum et al., 1990). Anti-viral activity, albeit to Plant viruses (TMV) has been reported in two proteins CIP-29 and CIP-34 isolated from the leaves of *Clerodendruminerme* Gaertn (Prasad, Srivastava, & Verma, 1995). What is interesting is that these proteins, which have exhibited antagonistic properties against the plant viruses have been found to function as Ribosome Inhibitory Proteins (RIPs). There is therefore a great scope – and given the fact that there is a greater need for anti-virals – that plant proteins along with plant secondary metabolites be screened for their potential anti-viral properties.

Conflict of Interest: The Authors declare no conflict of interest.

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