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RESEARCH ARTICLE

IN-VITRO ANTI RABIES VIRUS ACTIVITY OF INDIAN MEDICINAL PLANTS

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ABSTRACT

Of the estimated 55000 cases of Rabies deaths in the world, 20000 occur in India. Even in the second decade of the 21st Century there is no chemotherapeutic available for Rabies, a disease that finds a mention in Ayurveda. The present study was undertaken to assess the in vitro anti rabies virus activity of *Mangifera indica* (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. BHK-21 cells infected with the Challenge Virus Standard (CVS) strain of the Rabies Viruswere treated with the methanol and protein extracts from the plants. Anti-Viral activity was assessed using a modified Rapid Fluorescent Foci Inhibition Test (RFFIT) and reduction estimating the reduction in relative viral load using qRT-PCR. Methanol extract from Eranda exhibited the most effective activity with an IC_{50} value of 19.70 µg/mL (SI=496.95). This was followed by methanol extract of Mango followed by methanol extract of Bael – IC_{50} of 24.66 µg/mL (SI=286.29) and protein extract of Bael at 39.37 µg/mL (SI=350.27). Methanol extract of Bael also exhibited a promising IC_{50} at 55.71 µg/mL (SI=203.37). Viral load estimation presented a similar picture with methanol extracts of the 3 plants and the protein extract of Bael exhibiting over 100-fold reduction in the viral RNA copies as compared to controls at 1000 µg/mL. AM, MI and MP exhibited over 30-fold reduction even at 10 µg/mL. In the light of the situation where thousands of people die every year due to rabies and since there is no known anti-viral, the above results present potential leads, which must be explored further for developing anti rabies anti virals.

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INTRODUCTION

Rabies is a viral disease which has been known for ages. The descriptions of this disease have been found in the Sushruta Samhita – one of the founding texts of Ayurveda. Presently rabies deaths across the globe ae estimated at around 55000 per year (Knobel *et al.,* 2005) of which 20000 deaths happen in India (Devleesschauwer *et al.,* 2016). Although there exists an efficient vaccine, which can also be administered after the exposure, once the clinical manifestation sets in, death is inevitable. The Milwaukee protocol for management of clinical rabies also hasn't produced encouraging results. A major lacuna has been the non-availability of a successful specific anti-viral. Ribavirin and interferon-α have proved to be disappointing agents for the therapy of rabies. There is insufficient evidence to support the continued use of ketamine or amantadine for the therapy of rabies.

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Minocycline or corticosteroids are contraindicated owing to concerns about aggravating the disease (Appolinario and Jackson, 2015). Hence, there is an urgent need for developing anti rabies anti-viral. *Mangifera indica* (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. Also, components from the leaves of these plants have exhibited neuroprotective activity (Asaduzzaman *et al.,* 2014; Kawpoomhae, Sukma, Ngawhirunpat, Opanasopit, and Sripattanaporn, 2010; Lee *et al.,* 2012). Therefore, the present study was conducted to assess the anti-viral effect of methanol and protein extracts from the leaves of *Mangifera indica* (Mango), *Aegle marmelos* (Bael) and *Ricinus communis* (Eranda) against the rabies 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 *Mangifera indica* (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.

Virus titration

RV CVS stocks were grown in BHK-21 cell lines, and titer of infectious virus was measured by median tissue culture infective dose (TCID50) titration using Immunofluorescent Assay (IFA). Briefly, serial 10-fold dilutions (10^{-1} to 10^{-7}) of virus in serum-free MEM were added onto the confluent monolayer in 96-well tissue culture plate and incubated for 72 h at 37°C with 5% CO_2. After incubation, the medium was decanted and the cells were fixed by adding chilled acetone (50 µL per well) and kept at 4°C for 30 min. After discarding the acetone, cells were stained by anti-rabies virus N protein polyclonal florescent-labelled antibody (1:20) for 30 min and then washed (3 times) with 0.01 M phosphate buffer saline (PBS), air dried, and visualized under an inverted fluorescence microscope. The titer was calculated using Reed and Muench method and expressed as $TCID_{50}$.

***In vitro* cytotoxicity assay**

The*in vitro* cytotoxicity (CC_{50}) assay of the plant extracts was performed by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetra zolium bromide (MTT) assay. Briefly, BHK-21 cells were trypsinized and cultured onto 96-well plate at the density of 0.2×10^6 cells/ml. Once the monolayer is formed after 24 h, different concentrations (100-0.01 mg/mL)the samples (Soxhlet and protein) extracts were serially diluted (10 fold) in DMEM (without phenol red) and added to each culture wells in triplicate and incubated at 37°C with 5% CO_2 for 24 h. After incubation, the medium with plant extracts were removed and 10% of 5 mg/mL MTT (100 µL) was added to each well onto the cells in 96-well plate and incubated for 3 h at 37°C. After incubation, MTT (100 µL) was removed and the crystal formed (formazan) was solubilized by adding dimethyl sulfoxide to each well. The absorbance was read at 550 nm by an ELISA reader.

***In vitro* anti-rabies virus assay** (Mehta, Roy, and Chowdhary, n.d.) 50 µL of samples (1000 – 0.1 µg/mL) were mixed with a challenged dose of 10 $TCID_{50}$ RV CVS (50 µL) and incubated for 1 hr in 96 well tissue culture plate.

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)	Anisaldehyde sulphuric acid
Phenolics	Silica F_{254}	chloroform: methanol (9.5:0.5)	Anisaldehyde sulphuric 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

Cell line and virus

Baby Hamster Kidney (BHK-21) cell line was obtained from NCCS, Pune. The cells were cultured in minimum essential medium (MEM) with L-glutamine (2 mM), penicillin (100 IU/mL), streptomycin (100 µg/mL), and gentamicin (10 µg/mL) and supplemented with 10% fetal bovine serum (FBS). Flasks were incubated at 37°C, in 5% CO_2 incubator for 2–3 days until a monolayer was obtained. Rabies virus challenge virus standard (RV CVS) used in the present study was provided by the Department of Virology, Haffkine Institute. The *in vitro* cytotoxicity and *in vitro* antiviral assay were performed onBHK-21 cell line.

The reference serum (5 IU/ml) was serially diluted 2-fold from 1:00 to 1:2800 using MEM with 2% FBS in sterile tubes which serve as a positive control. BHK-21 cell line was trypsinized and 0.2×10^6cells/mL added to 96-well tissue culture plate. This was incubated at 37°C in a CO_2 incubator for 72 h. The medium was decanted and the cells were fixed by adding cold acetone (50 µL per well) and kept at 4°C for 30 min. After discarding the acetone, cells were stained by anti-rabies virus N protein polyclonal florescent-labelled antibody (1:20) for 30 min and then washed (3 times) with 0.01 M PBS, air dried, and visualized under an inverted fluorescence microscope.

Relative Viral load

Viral RNA was extracted from BHK-21 cells after*in vitro* antiviral assay on the 96-well plate using the spin column based QIAamp-Viral RNA Mini Kit (M/s. QIAGEN) as per the manufacturer's instructions. Reverse transcriptase real time-polymerase chain reaction (qRT-PCR) was carried out using a StepOne Plus Real-time PCR machine (M/s. ABI) using 2X PCR mix and Super-ScriptTMIIRT/Platinum Taq Enzyme mix (M/s. Takara) and rabies *N* gene primers (FWD:5'-TTGT(AG)GA(TC)CAATAT GAGTACAA-3'; REV: 5'-CCGGCTCAAACATTCTTCTTA-3'). The cycling condition for the qRT-PCR was 42°C for 5 min of reverse transcription followed by denaturation at 95°C for 10 s and PCR amplification (38 cycles) of 95°C for 1 min, 55°C for 1 min, and extension at 72°C for 1 min 30 s. Relative quantification of viral copy numbers was measured by Delta Delta C_t method(Livak and Schmittgen, 2001).

RESULTS

Phytochemical Profiling

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	+	+	++	++++	+++	++	-

In vitro cytotoxicity assay

Thein 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 11.33 mg/mL, 9.79 mg/mL and 7.0 mg/mL whereas Protein extract of Bael (MP), Eranda (RP) and Mango (MIP) were found to be 13.79 mg/mL, 4.94 mg/mL and 4.5 mg/mL respectively.

Table 3. Median Cytotoxic Concentrations of the samples on BHK-21 Cells

Median cytotoxic concentration (mg/mL)					
AM	RC	MI	MIP	MP	RP
11.33	9.79	7.06	4.5	13.79	4.94

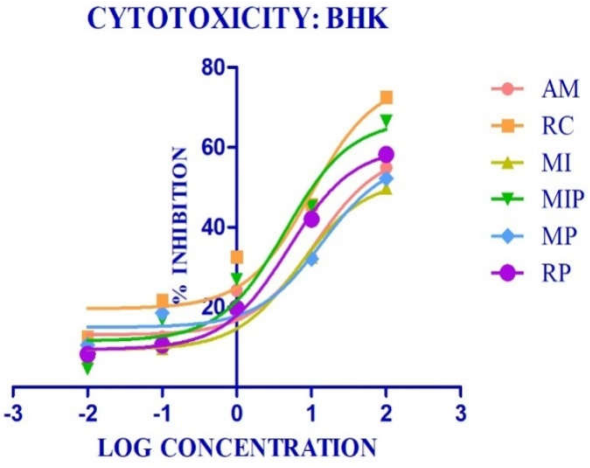

Figure 1. Graph depicting Cytotoxicity assay

In vitro anti-rabies virus assay

The in vitro antiviral activity of medicinal plant extracts (Bael, Eranda and Mango) were evaluated by RFFIT assay. The IC50 value and the selective index (SI) of Methanol extract of Bael (AM), Eranda (RC) and Mango (MI) were calculated and found to be 55.71 µg/ml (SI= 203.37), 19.70 µg/mL (496.95) and 24.66 µg/mL (286.29) respectively. whereas Protein extract of Bael (MP), Eranda (RP) and Mango (MIP) were found to be 39.37 µg/mL (SI=350.27), 310.30 µg/mL (SI=15.9) and 134.20 µg/mL (SI=33.53) respectively.

Table 4. Anti-Rabies Virus activity using modified RFFIT, results are expressed as Median Inhibitory Concentrations (IC50) of the Virus. The Selectivity Index (SI) is also mentioned. All concentrations are in µg/mL

	AM	RC	MI	MIP	MP	RP
IC50	55.71	19.70	24.66	134.20	39.37	310.30
SI	203.37	496.95	286.29	33.53	350.27	15.9

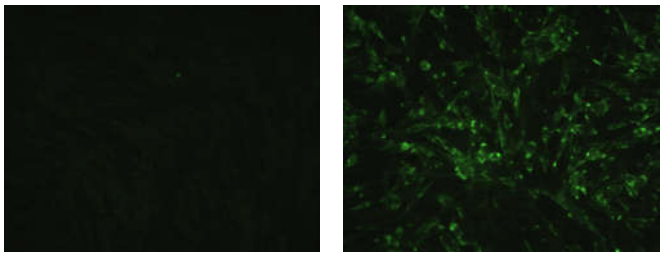

Figure 2. Fluorescent Foci Observed on BHK cells infected with the Rabies Virus (Right). Un-infected control cells (Left) did not exhibit any Fluorescent Foci

Table 5. Anti-Rabies Virus activity using qRT-PCR, results are expressed fold change w.r.t Virus Control

CONC. (µg/mL)	FOLD CHANGE					
	AM	RC	MI	MIP	MP	RP
1000	-166.57	-171.25	-286.03	-79.34	-188.71	-25.83
100	-74.54	-8.69	-52.71	-45.89	-121.1	-13.22
10	-39.95	-3.29	-32.45	-26.35	-37.27	-3.2
1	-8.11	-2.5	-3.29	-2.03	-8.11	-2.9

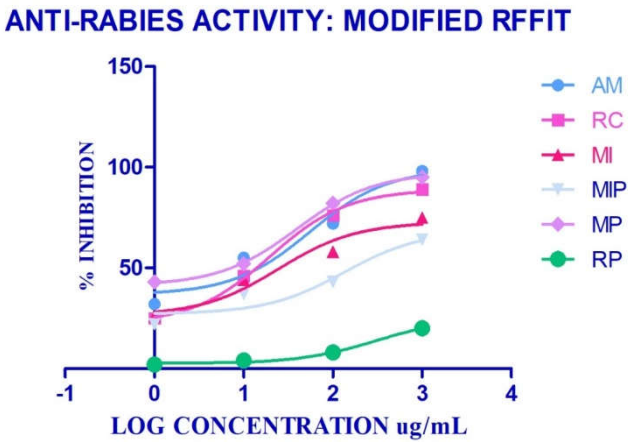

Figure 3. Graph of Anti-Rabies Virus activity using modified RFFIT

Relative Viral load

The in vitro antiviral activity of medicinal plant extracts (Bael, Eranda and Mango) were also evaluated using q-RT-PCR. At 1000 µg/mL Methanol extract of Bael (AM), Eranda (RC) and Mango (MI) exhibited around 166 fold, 171 fold and 286 fold

reduction in the viral load respectively, whereas Protein extract of Bael (MP), Eranda (RP) and Mango (MIP) reduced the viral loads by 188 fold, 25 fold and 79 folds respectively. AM, MI, MIP and MP reduced the viral loads by 39.95 fold, 32.45 fold, 26.35 fold and 37.27 folds respectively even at 10 µg/mL.

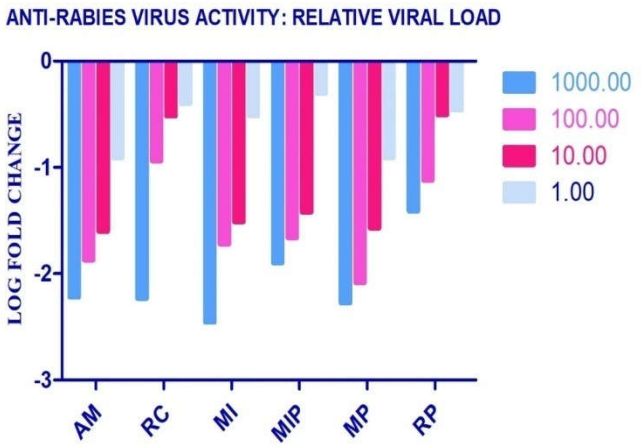

Figure 4. Graph of Anti-Rabies Virus activity using qRT-PCR

DISCUSSION

Rabies is of a particular importance in India, with over 20000 deaths reported annually (Devleesschauwer *et al.,* 2016). This too might be a case of under-reporting as the gold standard ante-mortem diagnostic test (RFFIT) is carried out in only 2-3 centres in India. Post-mortem diagnosis – although relatively easier (using PCR) – is not advised in most cases of idiopathic encephalitic deaths as very limited pathological autopsies are performed in India. Although the efficacy of anti-rabies immune-prophylaxis using Anti-Rabies Vaccine (ARV) and Rabies Immunoglobulin (RIG) is extremely good, there is lack of proper awareness in primary wound management and appropriate PEP even among the medical fraternity. This, along with the acute shortage of RIG's in the Indian market makes the situation of post exposure clinical management of rabies extremely grim(Mani, Anand, and Madhusudana, 2016). Experimental management strategies including the Milwaukee Protocol have appallingly limited if at all any clinical success. Moreover, there has been inadequate efforts at making a rabies specific anti-viral chemotherapeutic agent(Wilde and Hemachudha, 2015). Considering the high cost to income ratio of the rabies biologicals in the most affected parts of the world, a specific, effective and affordable chemotherapeutic agent against the rabies virus is the need of the hour. Large number of drugs in use in the clinics today are of phytological origins or primarily derived from plant sources (Atanasov *et al.,* 2015). 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 rabies and the influenza virus. Leaves of Mango (*Mangifera indica*), 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). The complete list of phytochemicals identified, was enlisted in Table 2 of the Results Section. Many other studies have reported similar phytochemical profiles from the leaves of these plants.

In-vitro cytotoxicity of the methanol extracts and protein extracts were assessed using the MTT assay on BHK-21 cells. The median cytotoxic concentrations were between 4.5 mg/mL for MIP to 13.79 mg/mL (MP) on BHK-21 cells. The Challenge Virus Standard (CVS-11) strain of the Rabies Virus was used in the study for assessing the anti-viral activity. Anti-viral activity against the rabies virus was evaluated using two methods; a modified RFFIT protocol and by assessing the relative change in viral nucleic acid copy number using qRT-PCR. Methanol extract from Eranda exhibited the most effective activity with an IC_{50} value of 19.70 µg/mL (SI=496.95). This was followed by methanol extract of Mango followed by methanol extract of Bael – IC_{50} of 24.66 µg/mL (SI=286.29) and protein extract of Bael at 39.37 µg/mL (SI=350.27). Methanol extract of Bael also exhibited a promising IC_{50} at 55.71 µg/mL (SI=203.37). Viral load estimation presented a similar picture with AM, RC, MI amd MP exhibiting over 100-fold reduction in the viral RNA copies as compared to controls at 1000 µg/mL. AM, MI and MP exhibited over 30-fold reduction even at 10 µg/mL. In the absence of an effective anti-rabies chemotherapeutic, it becomes necessary and imperative that search for novel anti-virals are expediated. Since phytochemicals have been the sources to severaltherapeutic agents against infectious and non-infectious diseases, they form an ideal starting point for such discoveries. The plants used in this study are long known in traditional and folk-lore medicine systems, especially in India. Further exploration using fractions from these extracts might yield critical lead compounds.

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