Use of rapid fluorescent focus inhibition test (RFFIT) for in vitro evaluation of anti-rabies activity

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Abstract Even in the twenty-first century, rabies remains one of the most dreaded diseases in many parts of the world. An effective chemotherapeutic still remains elusive. The present study was aimed at in vitro evaluation of crude extracts of *Allamanda cathartica* and *Cynodon dactylon* for their potential anti-rabies activity based on the principle of immunofluorescence. The extracts were tested for cytotoxicity and screened for the presence of phytochemicals. While *A. cathartica* extracts were found to be non-toxic, the CC₅₀ of *C. dactylon* (water and methanol) cold extracts were found to be 8.17 and 9.20 mg/mL respectively on BHK-21 cell line. Rapid Fluorescent Focus Inhibition Test (RFFIT) was used to evaluate anti-rabies activities of these plants against the rabies challenge virus standard strain. We observed 50% inhibition of 10 TCID₅₀ CVS at 5 mg/mL (IC₅₀) whereas fluorescence (no inhibition) was observed with *A. cathartica* extracts. The present study highlights the use of modified RFFIT as a method of choice for testing anti-rabies activity over assays based on evaluation of cytopathic effect.

Keywords Plant extracts · Anti-rabies · Immunofluorescence · RFFIT

Introduction

Rabies is caused by the highly neurotropic rabies virus that produces almost uniformly, fatal encephalitis in humans and most other mammalian species. Despite being 100% preventable nearly 20,000 deaths are reported annually from India alone (http://www.who.int/wer/2007/wer8249_50.pdf). No antivirals are currently available and hence development of new, cost-effective rabies biologics and antivirals is critical to prevent/reduce the disease burden. There is no established treatment available for rabies and management is only palliative. The successful recovery of a young girl from rabies using experimental approach involving use of drugs such as ketamine, midazolam, ribavirin and amantadine [25] has reinforced the need to search for effective anti-viral molecules with potential rabies inhibiting activity.

Rabies was very common in Ethiopia where traditionally a wide variety of plant-based remedies have been documented for its therapeutic use [15, 16]. One of the earliest attempts reports back to 1978, where Tsiang et al. [22] demonstrated inhibition of rabies virus replication in vitro using ammonium-5-tungsto-2-antimoniate (HPA 23) by plaque reduction and one step virus growth. More recently, Chavez and his colleagues evaluated anti-viral activity of 24 synthetic phenolic compounds against the Pasteur vaccine (PV) strain on McCoy cells and suggested the influence of free hydroxyl/ether groups for its anti-rabies activity [3]. Deressa et al. [5] evaluated crude extracts of *Silene maroselene* and *Salix subserrata* for anti-rabies activity in vivo while Muller et al. [12], tested methanolic extracts of flowers and leaves of *Allamanda schottii* using the viral cytopathic inhibitory assay. Methanolic leaf extract of *A. schottii* was reported to have a selective index (SI) of 5.6. It is evident that most of the in vitro antiviral

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screening assays for rabies virus till date have only been evaluated based on inhibition of cytopathic effect (CPE). However, rabies does not exhibit a distinct CPE when cultivated in tissue culture [11].

The present study was therefore designed to screen the crude leaf extracts of *Allamanda schottii* (Pohl) also known as *Allamanda cathartica*, and *Cynodon dactylon* (commonly known as Durva) for its in vitro anti-rabies activity using the Rapid Fluorescent Focus Inhibition Test (RFFIT), an assay independent of cytopathic effect (CPE).

Materials and methods

Collection and identification of sample

Fresh plant material of *A. cathartica* and *C. dactylon* were collected from a local nursery and Dadar flower market respectively in Mumbai. These were then identified in the Blatter herbarium at St. Xavier's College, Mumbai and authenticated to be *Allamanda cathartica* and *Cynodon dactylon*.

The plants parts were separately washed, shade dried and powdered in a grinder mixer. Approximately 25 g of powdered material was extracted in a Soxhlet extraction apparatus (continuous hot percolation) with 300 mL of two solvents—methanol (analytical grade) and water [8]. For preparation of cold extracts, 25 g of powdered material was extracted with 300 mL of methanol and water kept at room temperature for 24 h. Both soxhlet and cold extracts were then filtered through Whatman's No. 1 filter paper and the filtrates were separately concentrated to dryness using a rotary evaporator to remove the water and methanol. The crude plant extract obtained were weighed and stored at 4°C in refrigerator for further use.

Cell culture and viruses

Baby Hamster Kidney (BHK-21) cell lines were grown in Minimal Essential Medium (MEM) supplemented L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml) and gentamicin (10 µg/ml), and 10% Foetal Bovine Serum (FBS) (Gibco®, USA). The flasks were incubated at 37°C, in 5% CO₂ incubator for 2–3 days until a confluent monolayer is obtained [11]. Laboratory adapted rabies virus strain—Challenge Virus Standard (CVS) was used in the study.

Virus titration

CVS was grown on BHK-21 cell lines and titre of infectious virus was measured by 50% tissue culture infective dose (TCID₅₀)/Focus forming dose (FFD₅₀) titration.

Briefly, CVS was serially diluted (10⁻¹ to 10⁻⁶) in a serum-free-MEM and added onto the confluent monolayer in a 96-well tissue culture plate and incubated for 72 h at 37 °C with 5% CO₂. After incubation, the medium was decanted and the cells were fixed with chilled acetone (50 µL per well) for 30 min at 4°C followed by 1:20 FITC-tagged Polyclonal Direct Fluorescent Antibody (DFA) reagent (Merck Millipore) treatment (in dark) 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 titre was calculated using Reed & Muench method and expressed as TCID₅₀ [11].

In vitro cytotoxicity assay

The in vitro cytotoxicity (CC₅₀) assay of the plant extracts was performed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay on BHK-21 cell line. Briefly, effective concentrations of the plant extracts were prepared in MEM ranging from 100 to 0.1 mg/mL (tenfold dilutions) and were added on confluent cell monolayers of BHK-21 in 96-well plates. A cell control was maintained along with the test samples and the plates were incubated at 37 °C for 24 h. After incubation, medium was removed from all the wells and 100 µL of freshly prepared 0.5 mg/mL MTT (Sigma®) was added to each well and incubated for 4 h. After incubation, MTT (100 µL) was removed and the crystal formed (formazan) was solubilised by adding DMSO to each well and the absorbance was read on a multimode plate reader (Bio-tek®) at 540 nm [1, 10].

In vitro anti-rabies assay

Rapid Fluorescent Focus Inhibition Test (RFFIT) is one of the highly sensitive, specific and most commonly used test for estimation of rabies-virus neutralizing antibody. In the present study RFFIT was used to assess in vitro anti-viral activity of the aqueous and methanolic extracts of *A. cathartica* and *C. dactylon* against CVS by replacing test serum in the assay with various concentrations of the extract (5.0 mg/mL to 0.3 mg/mL). 50 µL of extracts was mixed with a challenged dose of 10 TCID₅₀ CVS (50 µL) and allowed to incubate for 1 h in 96 well tissue culture plate. Reference serum (5 IU/mL) was serially diluted two fold from 1:00 to 1:2800 using MEM with 2% FBS in sterile tubes served as positive control for the assay. BHK-21 cell line was trypsinized and 0.2 × 10⁶ cells/mL were added onto 96 well tissue culture plate containing the incubated mixture of virus and extract. The plate was incubated at 37°C in a CO₂ incubator for 72 h. Following which the medium was decanted and the cells were fixed with chilled acetone (50 µL per well) for 30 min at 4°C.

The fluorescence staining procedure followed was same as previously mentioned in virus titration [4, 11].

Graphs and statistical analyses

The data was analyzed, graphs were plotted and statistical analyses were done using GraphPad Prism 5 Software (GraphPad Software, San Diego, CA).

Virus titration and in vitro cytotoxicity assay

The virus was titrated on BHK-21 cells and the median tissue culture dose of 10^4 TCID₅₀/mL for CVS on BHK-21 cells was obtained as depicted in Fig. 1. A challenge dose of 10 TCID₅₀ was used for in vitro anti-rabies assay. Further, the extracts were also tested for their cytotoxic effects on BHK-21 cells, if any. While *Allamanda spp.* extracts were found to be non-toxic at all the tested concentrations, the aqueous and methanolic cold extracts of *C. dactylon* were found to have a CC₅₀ of 8.17 and 9.20 mg/mL respectively (Fig. 2).

Results and discussion

It is evident from literature that many traditional folk medicinal plants have strong anti-viral activities. Many Brazilian and South American plants have been evaluated for their anti-herpes and anti-rabies activity. One such plant is *Allamanda schottii*, also known as *Allamanda cathartica*, which has previously been demonstrated to have an SI of 5.6 against PV strain of rabies virus [13]. *Cynodon dactylon* or durva is used as a folk remedy for many medical ailments such as anasarca, alaculus, cancer, convulsions, epilepsy, hypertension, bronchitis, cough and diarrhoea. According to ayurvedic system of medicine it acts as an appetizer, antihelminthic, antipyretic, alexiteric agent and has a wound healing activity. In homeopathic

system of medicine it is used to treat all types of bleeding and skin diseases. It also has a CNS depressant activity [14, 21]. Based on the medicinal properties of these two plants, the present study was therefore designed to evaluate the anti-rabies activity of *A. cathartica* and *C. dactylon* using an immunofluorescence assay- RFFIT as a method of choice. The anti-viral activity of these extracts were tested against a laboratory adapted rabies strain-CVS.

Based on the CC₅₀ values, plant extract concentrations ranging from 5.0 mg/mL to 0.3 mg/mL were chosen to perform anti-rabies activity against a challenge dose of 10 TCID₅₀ on BHK-21 cells. Standard reference serum (5 IU/mL) titrated from 1:00 to 1:2800 and used as positive control for the assay. Absence of fluorescent foci indicated inhibition of virus replication/growth whereas presence of fluorescent foci indicated viral growth (Fig. 3). Fluorescence based RFFIT assay was used for testing anti-rabies activity of these plant extracts in vitro which in principle remains the same except that serum antibody is replaced by extracts.

The assay validation was based on the following: (a) Cell controls did not show any fluorescence (b) Virus controls did not show more than 50% fluorescent foci in the monolayer (c) Reference serum showed the expected 50% end-point dilution (Fig. 4). A minimum of ten microscopic fields were scanned for each dilution by two independent observers and staining intensity was graded as +4 (90–100% fluorescent monolayers), +3 (75% of monolayers with fluorescence), +2 (20–50% of monolayers with fluorescence) and +1 (<10% of fluorescence in monolayers) [11, 14]. We observed fluorescence (no inhibition of virus) at all concentrations with *A. cathartica*. The fluorescence intensity was found to be identical to the virus control i.e. +4. Therefore, it was inferred that at the employed concentrations, aqueous and methanolic (soxhlet and cold) extracts of *Allamanda cathartica* did not exhibit any rabies virus neutralizing activity. However 50%

Fig. 1 Titration of rabies CVS strain performed on BHK-21 cell line. Graph of virus log titre versus percent inhibition to determine the median tissue culture infectious dose 50 (TCID₅₀)

Fig. 2 In vitro cytotoxicity assay performed on BHK-21 cells with aqueous and methanolic (soxhlet and cold) extracts of *A. cathartica* and *C. dactylon*; S.W—soxhlet water (aqueous) extract, S.M—soxhlet methanolic extract, C.W—cold water (aqueous) extract, C.M—cold methanolic extract

Fig. 3 Evaluating anti-rabies activity using RFFIT in BHK-21 cells using (1:20) diluted FITC-tagged antibody specific against Nucleo-protein antigen (a) cell control with no fluorescence and (b) virus control with typical apple green fluorescence (colour figure online)

Fig. 4 Rabies reference serum (standard antibody) exhibiting 50% end-point dilution on titration against 10 TCID₅₀ CVS

inhibition of 10 TCID₅₀ CVS was observed at 5 mg/mL (IC₅₀) with *C. dactylon* but no inhibition was observed below 5 mg/mL i.e. from 2.5 to 0.3 mg/mL (Fig. 5).

Though Muller et al. detected anti-rabies activity with methanolic leaf extracts of *Allamanda schottii* when tested against PV strain of rabies virus on McCoy cells [24], our study exhibited contrasting results when tested against laboratory CVS strain on BHK-21 cells. This disparity may be attributed to geographical variations leading to a change in phytochemical profiles of the plant and therefore altered biological effects. There is documented evidence of environmental factors such as geographical location, fertility of cultivar, season of collection, etc., affecting the production of secondary metabolites that may be responsible for

Fig. 5 Graph depicting IC₅₀ of *C. dactylon* at 5 mg/mL

therapeutic properties exhibited by the plant. Ullah et al. [23], has previously demonstrated that extracts of plants obtained from higher altitudes and fertile cultivars were found to be have more effective antibacterial activities. However, while Muller & his colleagues inferred the results by visual scoring for inhibition of CPE, the present study was carried out with a purpose of evaluating anti-rabies activity independent of CPE. The assay was based on the principle of RFFIT wherein the test involves reaction of a mixture of dilutions of the test serum and a fixed amount of CVS, allowing for neutralization of the virus to occur, the extent of which is assessed by inoculating in cell culture and detecting the presence or absence of viral antigen by direct Fluorescent Antibody Test (FAT). In this case, the test serum sample was replaced by the plant extract.

The advantage of using a fluorescence based assay such as RFFIT over CPE inhibition is that it can be used to test anti-rabies activity of different molecules against all rabies and rabies related viral strains other than PV and on many different cell lines including cells of neuronal origin such N2a (neuroblastoma). Unlike most viruses that exhibit a distinct CPE when grown on monolayers of their susceptible cell line, for example HSV infected Vero cell line shows grape like clusters of round highly refractile cells [24], rabies virus infection/replication on a wide variety of cell lines is not accompanied by any clear definable CPE making it difficult to distinguish between controls and infected cells by simple microscopic observations. For instance, the rabies virus infected cells merely begin to age and detach more quickly from the supporting surface than uninfected control cells when cultivated on BHK-21 cell monolayers. However in McCoy cells, both fixed and street Rabies viruses cause changes within 24–72 h after infection, and is therefore generally preferred for anti-rabies assays [11]. More recently, Chavez et al. [2], studied anti-rabies activity using MTT assay but found it unsuitable for non-lytic viruses such as rabies.

Inhibition or reduction in the number of fluorescent foci has usually been the method of choice only to evaluate the anti-rabies effect of small interfering RNA (siRNA) in vitro [7, 18, 19]. The limitation however, is that it may give rise to inter-user variability error that may be overcome by scanning at least ten fields by two independent observers. In addition, it may not be possible to detect minimal inhibition or reduction in the number of viral particles that may be caused by plant extract/compounds under test at the various concentrations employed and hence immunofluorescence may also be complemented with methods of higher order of sensitivity such as chemiluminescence or molecular methods such as PCR.

Rabies continues to remain a major threat to public health causing numerous deaths around the world and

therefore the new research to find effective antiviral drugs continues. Small hairpin RNA (shRNA)/small interfering RNA (siRNA) are forerunners among the emerging therapeutic strategies being tested to inhibit viral replication both in vitro as well as in vivo [17, 20, 26]. Synthetic compounds such as HPA-23 [6] or phenolic compounds have also been shown to exhibit in vitro multiplication of rabies virus without affecting the viability of cells [9]. However, in the race to find new potential anti-rabies drugs, the paradigm appears to be shifting from synthetic/allopathic drugs towards ayurvedic/herbal medicines. Medicinal plants have a wide variety of chemical constituents that are known to inhibit DNA or RNA viral replication [12]. In addition, there are documented evidences of whole leaf or crude leaf extracts of plants such as *Commelina benghalensis* [18], *Ricinus communis* L [26], *Datura spp.*, *Solanum nigrum* L [17, 20, 26], etc. being used as ethnomedicines to treat rabies in the tribal as well as non-tribal areas of developing countries like India. An attempt can therefore, be made to identify and isolate the active components present in these plants and study the synergistic action of a combination of either two or more plants/plant extracts or both natural and synthetic compounds as potential anti-viral agents by using or developing methods independent of CPE inhibition.

Thus, the present study demonstrates the use of a CPE independent technique such as RFFIT for in vitro evaluation of anti-rabies activity of potential molecules. Considering the gravity of the disease in the world wide scenario, attempts at identifying novel anti-viral agents against the disease is imperative. Further there is an urgent need to develop better in vitro drug screening methods for anti-virals especially for fatal diseases like rabies. Thus immunofluorescence based assays such as RFFIT may be evaluated and used as standard protocols for such endeavors.

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