Antiviral potential of mangiferin against poliovirus

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Abstract
Poliovirus (PV), an enterovirus of the Picornaviridae family, is the agent of poliomyelitis, a devastating neurologic disease that may result in paralysis and even death. Mangiferin is the major constituent of Mangifera indica and has multiple pharmacological activities, including antiviral. This study evaluated the activity of mangiferin against poliovirus type-1 (PV-1). The compound exhibited the 50% cytotoxic concentration (CC50) > 2000 μg/mL in HEp-2 cell cultures, by the dimethylthiazolyl-diphenyltetrazolium bromide method (MTT). The 50% inhibitory concentration (IC50) was of 53.5 μg/mL, determined by the plaque reduction assay (PRA) with selectivity index (SI) > 37.4. We investigate the possible mechanism of action of mangiferin and a maximum inhibition of PV was found for virucidal and inhibition of adsorption assays, at all tested concentrations. The inhibition of viral protein synthesis was also demonstrated by immunofluorescence assay (IFA). These results suggested that mangiferin is an attractive candidate for the control of PV infection.

Keywords: Antiviral, Mangiferin, Poliomyelitis, Poliovirus.

1. Introduction
Poliovirus (PV), the etiologic agent of poliomyelitis, an enterovirus of the Picornaviridae family, is a non-enveloped virus with a positive single-stranded RNA genome [1;2]. PV is represented by three serotypes (PV-1, PV-2 and PV-3), however, no cross-reaction immunity is observed [1;3]. Poliomyelitis is an acute disease of the central nervous system that may result in permanent paralysis and death [4]. Its global incidence has decreased by 99%, and, in 2016, 37 cases were reported, oppositely to more than 350,000 in 1988. Currently, poliomyelitis is under control in most part of the world, due to massive vaccination program. However, despite intensive efforts to eradicate the virus, it remains endemic in Afghanistan, Nigeria and Pakistan [5].

The alternative strategy to control the disease is the use of drugs therapeutically, as well as, prophylactically. Molecules capable of inhibiting PV replication in cell culture have been described since the early 1960s [6;7]. A substantial number of compounds, including the naturals, have been reported as potent inhibitors of the replication of picornaviruses, in vitro.

Mangiferin is a xanthone glucoside obtained from various plants such as M. indica L. [8] and empirically, is used against diarrhea [9], vomiting [10], rheumatism [11], itch [12], asthma and gastric disorders [13]. Multiple pharmacological activities have been scientifically demonstrated, such as, antitumor [14-16], immunomodulatory [17], antidiabetic [18], antioxidant [19;20], antiallergic [21], anthelmintic [22], anti-inflammatory and antinociceptive [23;24]. The antibacterial (S. aureus, S. citreus, E. coli, B. pumilus, B. cereus, S. virchow, S. agona and K. pneumoniae), antifungal (S. cerevisiae, C. albicans, A. niger, A. flavus and T.
aurantiacus) and the antiprotozoal (C. parvum) activities have been reported [20;25;26]. Antiviral activity has also been shown against HIV-1 [14;27], HSV-1 and HSV-2 [28-30]. In addition, according to Anand et al. [31] and Gold-Smith et al. [32] mangiferin exhibits minimal toxicity in vitro and in vivo.

Although mangiferin is widely obtained from M. indica it was also described in several other plant species in the African and Asian continents [33]. The development of a new drug based on the principle of natural products may represent a great advantage, guaranteeing a lower cost with more efficiency and wide accessibility. Considering the need to develop new anti-PV drugs and the potentiality of mangiferin, this study investigated the anti-PV activity of this compound, in vitro.

2. Materials and methods
2.1 Compound

Mangiferin (Figure 1) extraction was performed at the Laboratório de Polímeros e Inovação de Materiais (LabPIM – UFCE) according to Muruganandan et al. [34], with minor modifications. Briefly, cold extraction was carried out with hexane (1:10; w/v) degreased 300 g of mango (Mangifera indica) peels. This was followed by the extraction of 100 g of dried and ground mango peels with Soxhlet extraction battery with methanol, for 5 days. The resulting methanolic extract was concentrated in a rotary evaporator at a maximum temperature of 65 ºC, for total solvent evaporation. The residue was resuspended with ethyl acetate, stored for 96 h at 5-10 ºC until the decantation of a yellowish precipitate, which was washed by light centrifugation with methanol.

![Figure 1: Chemical structure of mangiferin](image)

2.2 Cells and virus

HEp-2 cells (epithelial human larynx carcinoma cells, ATCC CCL-23) were grown in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (Invitrogen - Gibco, USA), 2 mM glutamine (Sigma Chem. Co., USA) and 100 μg/mL streptomycin, (Gibco BRL, USA), 100 IU/mL penicillin (Novafarma Ind. Farm., BR), and 2.5 μg/mL of amphotericin B (Meizler Biopharma S/A, BR).

PV-1 (ATCC VR-1562) was propagated in HEp-2 cells and stored in aliquots at -20°C with 10% glycerol. The virus titer was determined by plaque assay.

2.3 Cytotoxicity assay

Mangiferin cytotoxicity was evaluated by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide). Briefly, HEp-2 cell cultures grown at approximately 70% confluence in 96-well microplates (TPP, Switzerland) were treated with varying concentrations of the compound (31.25 to 2000 μg/mL) and maintained at 37 ºC with 5% CO2, during 72 h. The overlay medium was replaced with 10 μL of the MTT reagent (1.25 μg/mL) and incubated, under the same conditions, for 3 h. This was followed by the addition of 90 μL of the MTT solubilization solution (10% Triton X-100 in acidic isopropanol – 0.1N HCl) and after 15 min, under agitation, the optical density (OD) read at 570 and 690 nm. The percentage of cell viability (% CV) was calculated by the formula % CV = (At/Ac) x100, where At and Ac refer to the OD of test substance and control (untreated cells), respectively [35]. The 50% cytotoxic concentration (CC50) was calculated by linear regression analysis, as the concentration of the substance capable of reducing the OD of MTT product by 50% in comparison to control.

2.4 Plaque reduction assay

The compound antiviral activity was determined by plaque reduction assay (PRA) according to Espada et al. [36], with minor modifications. Briefly, HEp-2 cells grown at confluence in 24-well plates (TPP, Switzerland) were infected with PV-1 (103 PFU/mL) and treated with varying concentrations of the compound (200, 100, 50 and 25 μg/mL), added at different times (time-of-addition assay): treatments before (-1 and -2 h), during (0 h) and after (1, 2, 4, 8 and 12 h) infection. The cell cultures were overlaid with nutrient agarose (DMEM 2x1.8% agarose [v/v] containing 25 mM MgCl2). After 40 h of incubation at 37 ºC in 5% CO2 the cells were fixed and stained with 0.5% crystal violet in 20% ethanol. The plaques were counted and the percent of viral inhibition (%VI) calculated as [1 - (Vd/Vc)] x 100, where Vd and Vc refer to the number of plaques in the presence and absence of the compound, respectively [37]. The minimal concentration of the compound required to reduce 50% of plaque numbers (IC50) was calculated by regression analysis of the curve generated by plaque reduction assay. The selectivity index (SI) was expressed by the ratio of CC50/IC50.

PV-1 was treated with human alfa-2B interferon (Meizler Biopharma S/A, São Paulo, SP, BR), as positive control, at the concentration of 10,000 IU/mL.

2.4.1 Virucidal assay

A suspension PV-1 was incubated with DMEM containing the same concentrations of the compound (v/v), as in PRA, for 1 h at 37 ºC. Mangiferin concentrations with
virus were diluted 10-fold, added to cell culture (0.1 mL/well) and incubated for 1 h, and submitted to PRA [38].

2.4.2 Inhibition of adsorption assay

Briefly, cell monolayer was pre-incubated at 4 °C for 30 min and inoculated with virus simultaneously in absence and presence of the compound, at the same concentrations used before. After 80 min of adsorption period at 4°C, the cells were washed three times with cold PBS to remove the non-adsorbed virus, and, after incubation, PRA was performed [39].

2.5 Immunofluorescence assay

For immunofluorescence assay (IFA), HEp-2 cells grown in 24-well plates with cover slips were infected with PV-1 and treated with test substance at the concentrations of 200 - 25 μg/mL, at the time zero of infection. Twenty-four hours post infection, the cells were washed with Tween-20 PBS, fixed with cold acetone (-20°C) and blocked with 2% powdered skim milk PBS. Subsequently, rabbit anti-PV-1 serum (INCQS, Fiocruz, BR) was used, followed by washings and incubation with FITC conjugate goat anti-rabbit IgG (Sigma Chem. Co., USA). The cells were examined in a Zeiss fluorescence microscope (Zeiss Axio Imager A1) and 100 cells/coverslips were scored and the percentage of fluorescent cells inhibition calculated [40].

2.6 Statistical analysis

ANOVA followed by Tukey’s test (BioEstat 5.0 for Windows XP, 2007) were applied to determine the difference among experiments with the compound and control groups. Values of p < 0.05 were considered significant.

3. Results

The mangiferin showed no morphological changes in HEp-2 cell up to the highest concentration tested and presented a CC50 higher than 2000 μg/mL, by MTT assay.

The best inhibition of PV-1 replication was found when the compound was added simultaneously to the infection (time 0 h) with a VI of 97.8%, at the highest tested concentration (200 μg/mL), followed by 84.2% and 57.1% inhibition at concentrations of 100 and 50 μg/mL, respectively. The lowest inhibition (10.2%) was found at 25 μg/mL, showing a dose-dependent inhibition, at time 0 h treatment. The IC50 was 53.5 μg/mL and the SI >37.4. For the other time-of-addition protocols, at 200 μg/mL, mangiferin presented VI greater than 50%, 74.7% and 66.3% for 4 h and 12 h post-infection (pi) times, respectively. At the time 12 h, that inhibition was maintained up to the concentration of 100 μg/mL, with a VI of 62.7%. An inhibition of about 30% was found for times 1 h and 2 h, at concentrations of 100 and 200 μg/mL. The lowest inhibition was found at 8 h pi, with VI of 24.3% and for the pretreatments at -1 h and -2 h, with the VI of 18% and 9%, respectively, at the highest concentration tested (200 μg/mL). The highest antiviral activity was found for the virucidal and inhibition of adsorption assays with VI of 100%, at all tested concentrations (Figure 2).

Interferon, simultaneously tested as positive control, inhibited 100% of PV-1 replication at 10,000 IU/mL.

Figure 2: The inhibition of PV-1 by mangiferin for the protocols of time-of-addition, virucidal (V) and inhibition of adsorption (IA) in HEp-2 cell cultures by plaque reduction assay, at the indicated concentrations (200 a 25 μg/mL). The percentage of viral inhibition (%VI) was determined in comparison to controls and the results are expressed as mean ± SD of triplicate independent experiments.
The IFA, performed at time 0 h treatment, showed a dose-dependent inhibition of fluorescent cells (Table 1).

Table 1: The effect of mangiferin in the synthesis of PV-1 protein in HEp-2 cells by immunofluorescence assay. The compound was added at time zero of infection, at the indicated concentrations. The percentage of viral inhibition (%VI) was calculated with respect to untreated infected cells. The experiments were carried out in duplicate.

| Concentrations (μg/mL) | %VI   |
|------------------------|-------|
| 25                     | 46.6  |
| 50                     | 65    |
| 100                    | 74.4  |
| 200                    | 91.6  |

4. Discussion

The search aimed at the development of new compounds to control PV infection has been widely encouraged notwithstanding the use of vaccine. However, the restriction of the circulation and transmission of the oral vaccine virus strains are taken as the main concern, paradoxically to the concept when OPV was launched. Moreover, the minimization of the risk of the disease in endemic countries and the effort to avoid importation of virus where the disease is under controlled, justify the sought for the development of new anti-PV compounds. Anti-PV drugs would be highly indicated for immunocompromised who excretes the virus for long period, preventing exposure to nonimmune individual. Their use would be also recommended themselves in disease outbreaks, and, subsequently in the post-eradication era or in conjunction with inactivated vaccine [41].

In the present study, we demonstrated that according to CC<sub>50</sub>, IC<sub>50</sub> and SI that the mangiferin was effective against poliovirus replication at low concentrations (IC<sub>50</sub> = 53.5 μg/mL) and far from the maximum toxic dose tested (CC<sub>50</sub> > 2000 μg/mL), with SI > 37.4. In addition to presenting low toxicity, it is thought that natural products can generally exert biological activities, such as antiviral, by more than one mechanism of action. This possibility was analyzed under preestablished protocols. Initially, mangiferin was added simultaneously with virus (time 0 h) and resulted in a high % VI suggesting an action of the compound in different stages of poliovirus infection, such as, adsorption, penetration, replication, release and also virucidal activity.

In the other protocols of the time-of-addition assay, a weak inhibition was found for pretreatment at times -1 and -2 h, compared to later stages (4 h and 12 h post-infection), at the highest concentration tested, indicating an inhibition in steps such as transcription, translation, assembly/maturation, and release of the viral particle by the drug. Inhibition of protein synthesis, observed by IFA, also reinforces this suggestion. In corroboration with this result, Zhu et al. [30], evaluating the activity of the compound against herpes simplex virus-2 (HSV-2), an enveloped DNA virus, also suggested an inhibition in late stages of herpes virus replication.

In addition to the action of mangiferin in the late stages of viral replication, our results also demonstrated a strong virucidal and inhibition of viral adsorption activities at very low concentrations of mangiferin with 100% of VI at concentration as low as 25 μg/mL. The virucidal activity is very advantageous because viruses is inactivated before entry the cell and, obviously, avoids dissemination in the host, and further, contribute to the elimination of the virus in the environment, essential strategy for the control of the disease. Being PV non-enveloped, this action may be related mainly to changes in the capsid leading to loss of viral infectivity [42]. The combined use of two or more antiviral drugs, including anti-PV, has been recommended as a strategy for controlling viral diseases and reducing the emergence of resistant strains [43;44].

5. Conclusion

Therefore, we infer that mangiferin is a potential candidate for the control of PV infection due to the varying mechanisms of action, alone or in association with other drugs.

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