SEARCHING FOR A METHODOLOGY TO ASSESS *IN VITRO* ANTIVIRAL ACTIVITY PHOTOSENSITIZED

Mugas M.L[1], Konigheim B.[2], Aguilar J.[2], Marioni J.[1], Comini L.[1], Contigiani M.[2], Cabrera J.[1], Núñez Montoya S[1].

[1]IMBIV-CONICET - Farmacognosia, Dpto. Farmacia, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba. Ciudad Universitaria, Córdoba, X5000HUA, Argentina.

[2]Instituto de Virología (InViV), Facultad de Ciencias Médicas, Universidad Nacional de Córdoba. Ciudad Universitaria, Córdoba, X5000HUA, Argentina.

INTRODUCTION

Our research group studies the *in vitro* activity of photosensitizing anthraquinones (AQs), purified from a phototoxic plant, against several viruses.

Previously, we have demonstrated that these AQs exhibit virucidal effect against Herpes Simplex Virus Type-1 (HSV-1) and Junín Virus (JUNV), that means they inactivated viral particles before enter into the host cell, and even this inhibition was increased by light (photo-stimulation) (1,2).

To study the *in vitro* antiviral activity of these AQs (inside the infected cells, at some stage of viral replication), which can also be photostimulated, an appropriate methodology is needed.

OBJECTIVE

The aim of this work was to assess two techniques, the Neutral Red uptake assay (NR) and the test of plaque forming units reduction (PFU), establishing besides the conditions necessary to quantify the *in vitro* antiviral effect photosensitized.

MATERIALS AND METHODS

An extract enriched in AQs (3) was assayed in order to ensure a photostimulated effect against HSV-1. Seven concentrations were tested (≤CC_{50}), which were estimated from the curve of cellular viability vs. concentrations of extract on Vero cells, by the NR assay. The antiviral effect was evaluated by perfoming both aforementioned techniques in Vero cells, under two simultaneous conditions: darkness and irradiation (actinic lamp 380-480 nm, Philips TL/03). The following variables were evaluated: culture medium during irradiation, irradiation time on virus-cells-extract, culture medium post-irradiation and incubation time.

RESULTS AND DISCUSSION

1. Culture medium during virus irradiation

Two culture medium: Minimum Essential Medium (MEM) and Phosphate Buffer Saline (PBS) were used to suspend the viral particles (1 x 10^{-4}) during irradiation. Thus, the impact of the irradiation time on the virulence of the virus in different culture media was studied, knowing that one has antioxidants (MEM) and the other not (PBS). Both alternatives were compared to the virus control (VC) in the dark .

The VC were similar in both culture media (Fig.1). Viral particles in MEM were mildly inhibited with 15 min of irradiation. With 30 min of irradiation, the action of light began to be important because the amount of virus decreased (increased the cellular viability, CV). By contrast, no significant differences between the different irradiation times and the respective control using PBS were observed; so the latter would be the optimum culture medium to use during irradiation.

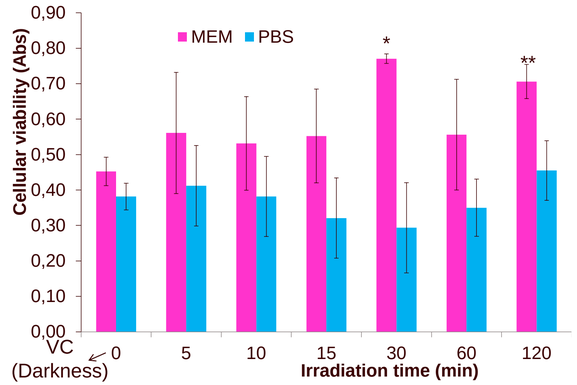


Figure 1: Viability of infected cells in different growth media with different irradiation times.

2. Irradiation time on cells-extract

The effect of light on a monolayer of Vero cells (2 x 10^5, 24 h in MEM) versus time proved that 15 min irradiation in the presence of phosphate buffer saline (PBS) caused no significant morphological changes with respect to control in darkness.

3. Incubation time and culture medium post-irradiation

The incubation time for HSV-1 is 72 h, according to the protocol of Virology Institute.

Eight culture media were evaluated to choose one for using post-irradiation (during incubation), which composition is shown in Fig. 2.

Culture medium components	Culture Medium post irradiation evaluated							
	1	2	3	4	5	6	7	8
MEM 1X 2% SFB, 1% DMSO	1	½						
PBS 1% DMSO		½	½	½	½	½	½	
MEM 4% SFB			½					
MEM 2X 2%SFB				½				
PBS 2% SFB + 1%ATB + 1%DMSO					½			
PBS 4% SFB + 1%ATB + 1%DMSO						½		
Isotonic glucose 5%							½	
PBS (1% DMSO) + Isotonic glucose 5% (10:1)								1

Figure 2: Composition of evaluated culture media to be used post-irradiation of infected cells.

RESULTS AND DISCUSSION

Fig. 3 shows the viability of infected cells which were incubated 72 h with different culture media, after being irradiated 15 min in PBS. Although no significant differences between 1 (culture medium chosen as VC) and 2-6 were observed, the culture medium 2 was chosen because it was the only one that did not affect the viability of uninfected cells (Cellular Control, CC). By contrast, 3-6 decreased the CV of CC (data not shown), whereas 7 and 8 showed an inhibitory effect on the virus, so that they were discarded.

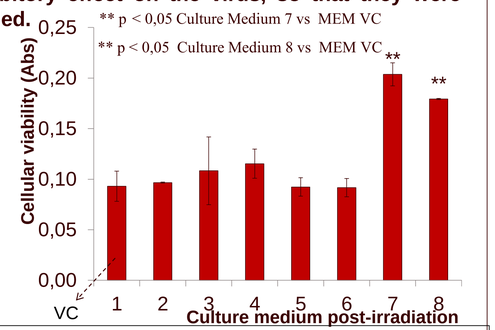


Figure 3: Viability of infected cells with HSV-1, incubated in different culture media post-irradiation.

4. Techniques

Based on these previous results, two protocols which use two different techniques were developed: Neutral Red uptake assay (NR) and reduction of plaque forming units test (PFU). The aim was to discriminate which method is best for studying the *in vitro* antiviral activity photosensitized. For this, seven concentration (≤ CC_{50}) were tested on the viral model chosen. The results obtained are shown in Figures 4, 5.

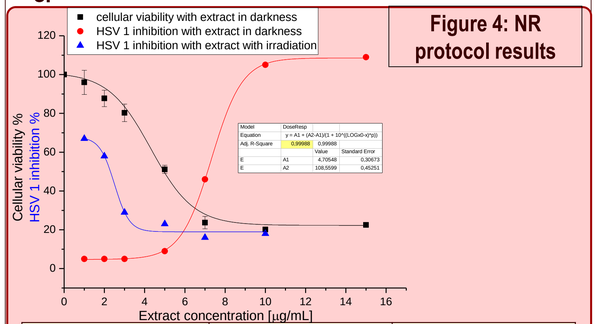


Figure 4: NR protocol results

CC_{50}	SubTC	MNCC
(68,66 ± 0,20)µg/mL	(29,82 ± 0,20)µg/mL	(13,77 ± 0,10)µg/mL

Extract concentration (µg/mL)	Cellular viability in darkness (%)	Viral inhibition with irradiation(%)
1	98,00 ± 0,41	66,93 ± 0,41

RESULTS AND DISCUSSION

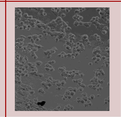
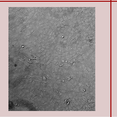
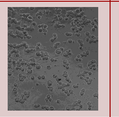
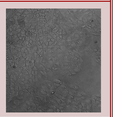
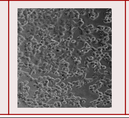
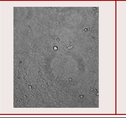
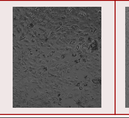
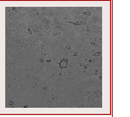
	Viral control	Cellular control	Cell + virus + extract	Cell + extract
Darkness				
Irradiation				

Figure 5: RN protocol, cytopathic effect.

Fig. 4 shows that the NR protocol allow determining the inhibition percentage, calculated as has been described by Semple et al (4). These results can be corroborate by the cytopathic effect observation by means of optic microscopy (Fig. 5). The PFU protocol (data no shown) only allowed us to observe the qualitative cytotoxicity caused by the photostimulated extract despite being a quantitative technique.

CONCLUSION

The optimal conditions for both methodologies were: PBS 1% DMSO (culture medium during irradiation), 15 minutes (irradiation time on virus-cells-extract), MEM 1x (2% FBS and 1% DMSO) – PBS (1% DMSO) [1:1] (culture medium post-irradiation), and 72 h incubation. The protocol that uses the NR assay was chosen to evaluate the *in vitro* antiviral effect photostimulated because it allowed determining the inhibition percentage, which could not be estimated by the PFU test, since it only showed the qualitative toxicity of the photostimulated extract. Besides, during the NR assay, the cytopathic effect (microscopic observation of morphological alterations in cells) could be simultaneously assessed, which is very important because it allowed discriminating the responsible agent of this effect.

LITERATURE CITED

1. SC Núñez Montoya, JJ Aguilar, BS Konigheim, MS Contigiani, JL Cabrera. Fotoestimulación de la actividad antiherpética de antraquinonas naturales. XVI CONGRESO ITALO-LATINOAMERICANO DE ETNOMEDICINA. (SILAE). La Plata, 04-08/09/2007. Arg.
2. MD Beranek, BS Konigheim, JJ Aguilar, MS Contigiani, JL Cabrera, SC Núñez Montoya. Efecto de la fotoestimulación de antraquinonas naturales sobre el virus Encefalitis San Luis. XXX REUNIÓN CIENTÍFICA ANUAL DE LA SOCIEDAD ARGENTINA DE VIROLOGÍA. Villa Giardino, 8-11/12/2010. Córdoba, Arg.
3. Konigheim B, Beranek M, Comini L, Aguilar J, Marioni J, Cabrera J, Contigiani M, Núñez Montoya S . In vitro antiviral activity of Heterophyllaea pustulata extracts. Nat. Prod. Commun 7:1025-28.
4. Semple S, Reynolds G, O'Leary M, Flower R. Screening of Australian medicinal plants for antiviral activity. Journal of Ethnopharmacology 60:163-72.