**SUPPLEMENTARY MATERIAL.**

# A quantitative PCR-based measurement for antiviral activity screening of medicinal plants against Herpes Simplex 1.

Authors:

*Gabaglio, S.[1#]; Alvarenga, N.[2#]; Cantero–González, G.[1#]; Degen, R.[4]; Ferro, E.A[2]; Langjahr, P.[1]; Chnaiderman, J.[3]; Sotelo P. H.[1*]*

[1]*Departamento de Biotecnología, Facultad de Ciencias Químicas, Universidad Nacional de Asunción, Paraguay,*

[2]*Departamento de Fitoquímica, Facultad de Ciencias Químicas, Universidad Nacional de Asunción, Paraguay,*

[3]*Programa de Virología, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Chile*

[4]*Departamento de Botánica, Facultad de Ciencias Químicas, Universidad Nacional de Asunción, Paraguay,*

[#] Contributed equally to this work

[*]Corresponding authors.

Dpto. de Biotecnología, Facultad de Ciencias Químicas, Universidad Nacional de Asunción, Campus Universitario, San Lorenzo, Paraguay. (595-21) 585 564; *phsotelo@qui.una.py.

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**Abstract**

Herpes simplex virus 1 is one of the most prevalent pathogens worldwide. Strains resistant to current treatment have been reported, so it is necessary to search for new antiviral molecules. The most common method to quantify antiviral activity from natural products is the plaque reduction assay, a technically demanding method. In order to provide a simple alternative to this method, we have established a procedure for viral quantification by qPCR, and coupled with a cytotoxicity evaluation system using resazurin. In this way, it is possible to obtain both the estimation of cytotoxicity and the antiviral activity simultaneously, allowing rapid screening of plant extracts. Ten out of twenty-eight Paraguayan medicinal plant extracts evaluated using this method showed antiviral activity, and the $EC_{50}$, $CC_{50}$, and SI values were calculated for each extract. Our experience supports the employment of the described method for a rapid identification of plant extracts with antiviral activity.

**Keywords: antiviral screening, qPCR, HSV-1, plant extracts**

**Experimental procedure**

Virus and cells

African green monkey kidney cells Vero CCL-81 were cultured in DMEM (GIBCO) 7.5% FCS (GIBCO). HSV-1 strain F was grown in Vero cells and viral stocks were quantified by plaque assay (Ejercito, Kieff, & Roizman, 1968).

qPCR methods

For the lysis of viral supernatants, 270 µL of lysis buffer (200 µg/mL proteinase K, 0.45% of IGEPAL, 0.45% Tween 20, Tris-HCl 10 mM, pH:8) were added to 30 µL of each supernantant and incubated for 90 min. at 56 °C, and then at 95 °C for 10 min (Nguyen, Deback, Malet, Bonnafous, & Agut, 2006). Afterwards, qPCR was performed using 2.5 µL of lysate. All qPCR reactions were performed by dye-based quantification, for which 10 µL reaction mix was prepared with 2.5 µL of each template, 5 µL SsoAdvanced Universal SYBR Green Supermix (BIORAD), 0.5 µL of each 10 mM primer FW (5'-GTCGCCTTACGTGAACAAGAC-3') and RV (5'-GTCGCCATGTTTCCCGTCTG-3') (Garvey, Mcgowin, & Foster, 2014). The amplification step was carried out in a LightCycler Nano (Roche), using the following program: initial denaturation at 95 °C for 5 min, 35 cycles of two-stage amplification: 95 °C for 15 s, 60 °C for 30 s. Finally, a denaturing curve of amplified products was made starting at 65 °C for 20 s, then increasing by 0.1 °C/s up to 95 °C, and finishing at 95 °C for 20 s. Reactions without any template were performed as negative controls. A calibration curve was made using purified amplicon log viral genome copies (VGC) vs Cq.

Plant material

Plants were collected from different departments of Paraguay, and identified by botanist Rosa Degen. Voucher specimens were deposited in the Herbarium FCQ, from the Department of Botany, Facultad de Ciencias Químicas, UNA for indexing purposes.

Plant material was air dried and grounded to fine powder in a knife mill prior to extraction. Extracts were obtained by ultrasound-assisted maceration, suspending the powder in HPLC-grade methanol (1.5 L per 200g) and sonicated it for 30 minutes three times at intervals of 15 minutes. The macerate was left overnight and filtered the day after through qualitative paper. Fresh solvent was added and the extraction cycle was repeated for two more days. The filtrates were reunited and the solvent was eliminated using a rotary evaporator (RVO 400 SD Boeco, Germany) to obtain the crude extract. The extracts were weighed and the yield was calculated with regard to the dry initial material (Table S3).".

Antiviral evaluation

Vero cells were seeded into 96 wells plates at $1\times10^4$ cell/well in 100 µL of DMEM 7.5% FCS. On the next day, cells were washed and incubated with 50 µL of virus at MOI 1.5 diluted in DMEM 2% FCS. Then, after one hours, the virus was removed and 100 µL of DMEM 2% FCS with or without plant extract was added. Each extract was analyzed by triplicate. Control infected cells were treated in the same conditions and incubated with DMSO. Uninfected control cells were treated in same conditions without addition of virus. After 72 h.p.i. 30 µL of supernatant was lysed and analyzed by qPCR as previously described.

The antiviral activity was calculated as follows:

$$\%of \in hibition = 100\left(1 - \frac{VGCextract}{VGCDMSO}\right)$$

To calculate the $EC_{50}$ extracts, the samples were submitted to a two-fold serial dilution and analyzed as previously described.

Cytotoxicity evaluation

Vero cells were seeded in 96 wells plates at $1\times10^4$ cell/well in 100 µL of DMEM 7.5% FCS. On the next day, cells were washed and culture medium was replaced by 100 µL of DMEM 2% FCS with or without plant extract. After 72 h.p.i., resazurin was added to obtain a final concentration of 0.0015% per well. Following 3 hours of incubation, absorbances at 570 and 630 nm were measured.

To calculate the percentage of cytotoxicity, the following formula was used:

$$\%ofcitoxicity = 100\left(1 - \frac{Eox\lambda2.A\lambda1 - Eox\lambda1.A\lambda2extract}{Eox\lambda2.A\lambda1 - Eox\lambda1.A\lambda2DMSO}\right)$$

Where: Eox= molar extinction coefficient of oxidized resazurin

$\lambda1$ = 570 nm

$\lambda2$ = 630 nm

In the antiviral screening assay, the control cells without infection were used to analyze the cytotoxicity.

To calculate the $IC_{50}$ extracts the samples were submitted to a two-fold serial dilution and analyzed as previously described.

Table S1. Antiviral activity against HSV 1 and cytotoxicity of the plant extracts

| **Species** | **Cytotoxic activity (%) at 62.5 µg/mL** | **Antiviral activity (%) at 62.5 µg/mL** | **CC50** | **EC50** | **SI** |
|---|---|---|---|---|---|
| ***Acacia caven (Molina)* Molina** | 4.49 | 12.89 | n.a | n.a | n.a |
| ***Acanthospermum australe* (Loefl.) Kuntze** | 2.59 | 71.78 | 178.6 | 18.39 | 9.71 |
| ***Acanthospermum hispidum DC.*** | 5.24 | 79.71 | 255.6 | 1.94 | 131,75 |
| ***Aloysia gratissima* (Gillies & Hook. ex Hook.) Tronc. var. *gratissima*** | 0.24 | 5.50 | n.a | n.a | n.a |
| ***Aloysia virgata*(Ruiz & Pav.) Pers. var *platyphylla* (Briq.) Moldenke** | 7.01 | 43.96 | n.a | n.a | n.a |
| ***Amphilophium paniculatum* (L.) Kunth** | 85.38 | 2.77 | n.a | n.a | n.a |
| ***Annona emarginata* (Schltdl.) H. Rainer** | 7.8 | 94.90 | 127.1 | 4.47 | 28.4 |
| ***Austroeupatorium inulifolium* (Kunth) R.M. King & H. Rob.** | 0 | 5.67 | n.a | n.a | n.a |
| ***Baccharis dracunculifolia* DC.** | 13.85 | 79.79 | 175.3 | 8.01 | 21.88 |
| ***Baccharis notosergila* Griseb.** | 4.24 | 82.39 | 175.4 | 7,61 | 23.04 |
| ***Baccharis punctulata* DC.** | 38.61 | 14.94 | n.a | n.a | n.a |
| ***Baccharis trimera* (Less.)DC.** | 0.6 | 71.98 | 275 | 22.63 | 12.16 |
| ***Calea uniflora* Less.** | 2.82 | 0.00 | n.a | n.a | n.a |
| ***Centratherum punctatum* Cass.** | 81.13 | 3.18 | n.a | n.a | n.a |
| ***Croton paraguayensis* Chodat** | 1.76 | 8.11 | n.a | n.a | n.a |
| ***Chromolaena ivifolia* (L.) R.M. King & H. Rob.** | 0.9 | 21.04 | n.a | n.a | n.a |
| ***Lessingianthus niederleinii* (Hieron.) H. Rob.** | 3.89 | 69.25 | 385,5 | 8.66 | 44,51 |
| ***Lippia recolletae* Morong.** | 15.23 | 11.62 | n.a | n.a | n.a |
| ***Lippia origanoides* Kunth** | 17.09 | 18.07 | n.a | n.a | n.a |
| ***Phoradendron liga* (Gillies ex Hook. & Arn.) Eichler EK 149PE** | 13.53 | 10.39 | n.a | n.a | n.a |
| ***Pluchea sagittalis* (Lam.) Cabrera** | 26.96 | 15.95 | n.a | n.a | n.a |
| ***Pterocaulom angustifolium* DC** | 29.56 | 19.21 | n.a | n.a | n.a |
| ***Solanum americanum* Mill.** | 6.3 | 76.73 | 475 | 3.26 | 145,7 |
| ***Solanum comptum* C. V. Morton** | 0 | 80.05 | 1590 | 7.62 | 208.66 |
| ***Solanum sisymbriifolium* Lam.** | 6.41 | 92.36 | 2206 | 18.63 | 118.41 |
| ***Solidago chilensis* Meyen** | 15 | 8.08 | n.a | n.a | n.a |

| | | | | | |
|---|---|---|---|---|---|
| ***Stachytarpheta cayennensis* (Rich.) Vahl** | 11.24 | 8.84 | n.a | n.a | n.a |
| ***Tessaria dodoneifolia* (Hook. & Arn.) Cabrera** | 19.87 | 0.00 | n.a | n.a | n.a |

$CC_{50}$: Cytotoxicity Concentration 50%
$EC_{50}$: Antiviral Effective Concentration 50%
SI: Selectivity Index.
n.a: Not analyzed

Table S2. Vernacular names and popular use of plants showing anti HSV-1 activity

| **Species** | **Vernacular name** | **Popular use.** | **Administration routes** |
|---|---|---|---|
| ***Acanthospermum australe* (Loefl.) Kuntze** | tapekué | Anti-inflammatory. For washing ulcerous wounds. | Topical use, on the skin. Applied by gargle in the oropharyngeal mucosa. |
| ***Acanthospermum hispidum* DC.** | tororatí | Anti-inflammatory. | Topical use, on the skin. |
| ***Annona emarginata* (Schltdl.) H. Rainer** | aratiku'i | To treat tonsillitis. | Oral use in tea infusions. Topical use, on the skin. |
| ***Baccharis dracunculifolia* DC.** | chirca | Antiseptic, to treat colics. | Oral use in tea infusions. Topical use, on the skin. |
| ***Baccharis notosergila* Griseb.** | typychá guaikurú | For insects bites. | Topical use, on the skin. |
| ***Baccharis trimera (Less.) DC.*** | jaguarete ka'a | Digestive, emmenagogue. | Oral use in tea infusions. |
| ***Solanum americanum* Mill.** | arachichu | To treat Herpes zoster. | Topical use, on the skin. |
| ***Solanum comptum* C. V. Morton** | Not reported | Not reported. | Not reported. |
| ***Solanum sisymbriifolium* Lam.** | ñuatí pyta | Diuretic and hypotensive. | Oral use in tea infusions. |
| ***Lessingianthus niederleinii* (Hieron.) H. Rob.** | Not reported | Not reported. | Not reported. |

Table S3 Plants extract evaluated against HSV-1.

| Species | Family | Place of collection | Collector/ Voucher No | Extract yield (%) |
|---|---|---|---|---|
| ***Acacia caven (*Molina*)* Molina** | Fabaceae | Escobar, Paraguarí, Paraguay | R. Degen 4164 | 22.6 g (10.24) |
| ***Acanthospermum australe* (Loefl.) Kuntze** | Asteraceae | San Lorenzo, Central, Paraguay | R. Degen 4061 | 31.3 g (14.40) |
| ***Acanthospermum hispidum* DC.** | Asteraceae | Costa Pukú , Cordillera, Paraguay | R. Degen 4074 | 21.8 g (8.94) |
| ***Aloysia* gratissima (Gillies & Hook. ex Hook.) Tronc. v*ar. gratissima*** | Verbenaceae | Escobar, Paraguarí, Paraguay | R. Degen 4042 | 29.4 g (5.45) |
| ***Aloysia virgata* (Ruiz & Pav.) Pers. var *platyphylla* (Briq.) Moldenke** | Verbenaceae | Escobar, Paraguarí, Paraguay | R. Degen 4067 | 36.6 g (4.75) |
| ***Amphilophium paniculatum* (L.) Kunth** | Bignoniaceae | Caacupé, Cordillera, Paraguay | R. Degen 4224 | 63.74 g (7.89) |
| ***Annona emarginata* (Schltdl.) H. Rainer** | Annonaceae | San Lorenzo, Central, Paraguay | R. Degen 4064 | 30.4 g (15.83) |
| ***Austroeupatorium inulifolium* (Kunth) R.M. King & H. Rob.** | Asteraceae | San Lorenzo, Central, Paraguay | R. Degen 4079 | 11.9 g (4.91) |
| ***Baccharis dracunculifolia* DC.** | Asteraceae | San Lorenzo, Central, Paraguay | R. Degen 4272 | 15.35 g (6.90) |
| ***Baccharis notosergila* Griseb.** | Asteraceae | Presidente Hayes, Paraguay | R. Degen 4362 | 47.7 g (10.9) |
| ***Baccharis punctulata* DC.** | Asteraceae | Ñemby, San Lorenzo, Central, Paraguay | R. Degen 4302 | 24.12 g (6.96) |
| ***Baccharis trimera* (Less.) DC.** | Asteraceae | San Lorenzo, Central, Paraguay | R. Degen 4088 | 17.1 g (5.04) |
| ***Calea uniflora* Less.** | Asteraceae | Aguaity, Cordillera, Paraguay | R. Degen 4291 | 11.10 g (5.25) |
| ***Centratherum punctatum* Cass.** | Asteraceae | Paraguarí, Paraguarí, Paraguay | R. Degen 4044 | 34.4 g (5.23) |
| ***Croton paraguayensis* Chodat** | Euphorbiaceae | 6 de enero, Cordillera, Paraguay | R. Degen 4198 | 3.74 g (4.89) |
| ***Chromolaena ivifolia* (L.) R.M. King & H. Rob.** | Asteraceae | Aguaity, Cordillera, Paraguay | R. Degen 4016 | 18.2 g (6.30) |
| ***Lessingianthus niederleinii* (Hieron.) H. Rob.** | Asteraceae | Aguaity, Cordillera, Paraguay | R. Degen 4236 | 27.7 g (4.51 |
| ***Lippia recolletae* Morong.** | Verbenaceae | Ñemby, San Lorenzo, Central, Paraguay | R. Degen 4290 | 37.5 g (13.58) |
| ***Lippia origanoides* Kunth** | Verbenaceae | Tobatí,Cordillera, Paraguay | R. Degen 4257 | 50.25 g (8.76) |
| ***Phoradendron liga (*Gillies ex Hook. & Arn.) Eichler EK 149 PE** | Viscaceae | Ruta 14 de mayo, Paraguarí, Paraguay | R. Degen 4321 | 58 g (10.05) |
| ***Pluchea sagittalis* (Lam.) Cabrera** | Asteraceae | San Lorenzo, Central, Paraguay | R. Degen 4065 | 9.1 g (3.07) |
| ***Pterocaulom angustifolium* DC** | Asteraceae | Escobar, Paraguarí, Paraguay | R. Degen 4039 | 14.8 g (4.07) |
| ***Solanum americanum* Mill.** | Solanaceae | San Lorenzo, Central, | R. Degen 4347 | 22.3 g (4.34) |

| | | | | |
|---|---|---|---|---|
| | | Paraguay | | |
| ***Solanum comptum* C. V. Morton** | Solanaceae | Aguaity, Cordillera, Paraguay | R. Degen 4262 | 13.37 g (3.72) |
| ***Solanum sisymbriifolium* Lam.** | Solanaceae | Tobatí, Cordillera, Paraguay | R. Degen 4065 | 14 g (9.06) |
| ***Solidago chilensis* Meyen** | Asteraceae | Díaz Cué, Cordillera, Paraguay | R. Degen 4032 | 31.8 g (6.01) |
| ***Stachytarpheta cayennensis* (Rich.) Vahl** | Verbenaceae | Areguá, Central, Paraguay | R. Degen 4038 | 44.5 g (11.05) |
| ***Tessaria dodoneifolia* (Hook. & Arn.) Cabrera** | Asteraceae | Díaz Cué, Cordillera, Paraguay | R. Degen 4127 | 38.3 g (11.46) |

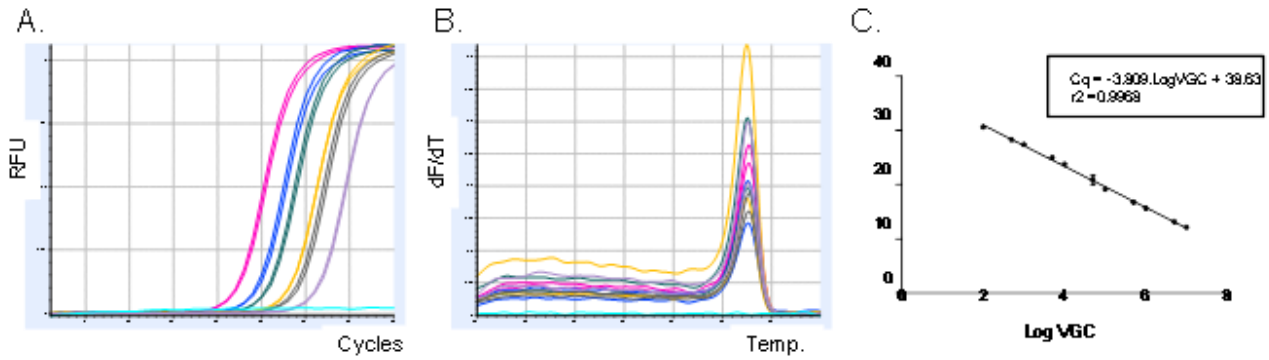


Figure S1: Calibration curve. A) Amplification curves. Dilutions of amplicon copies were amplified by qPCR. B) Melting curve, where the presence of a single amplicon is observed. C) Linear regression analysis of the data.

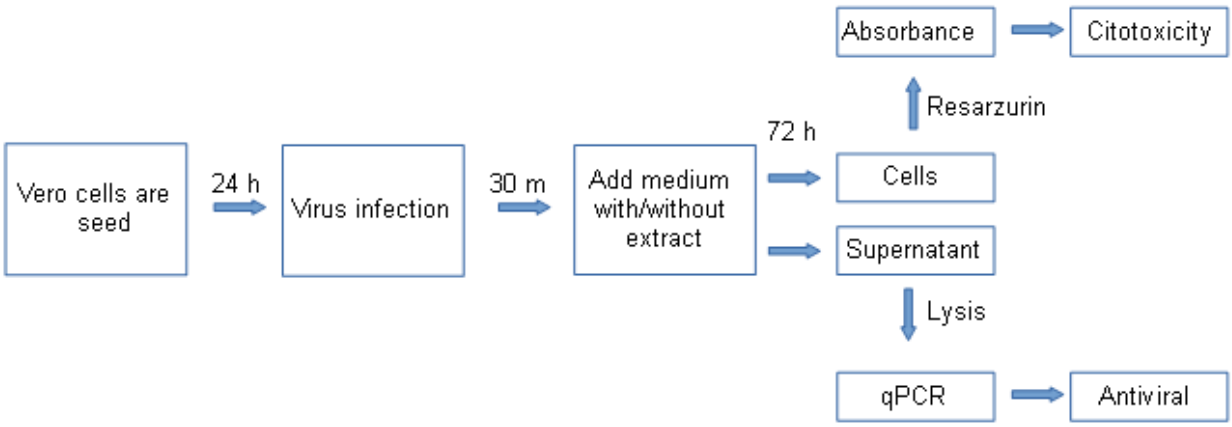


Figure S2: Schematic pipeline of cytotoxicity and antiviral assays.

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