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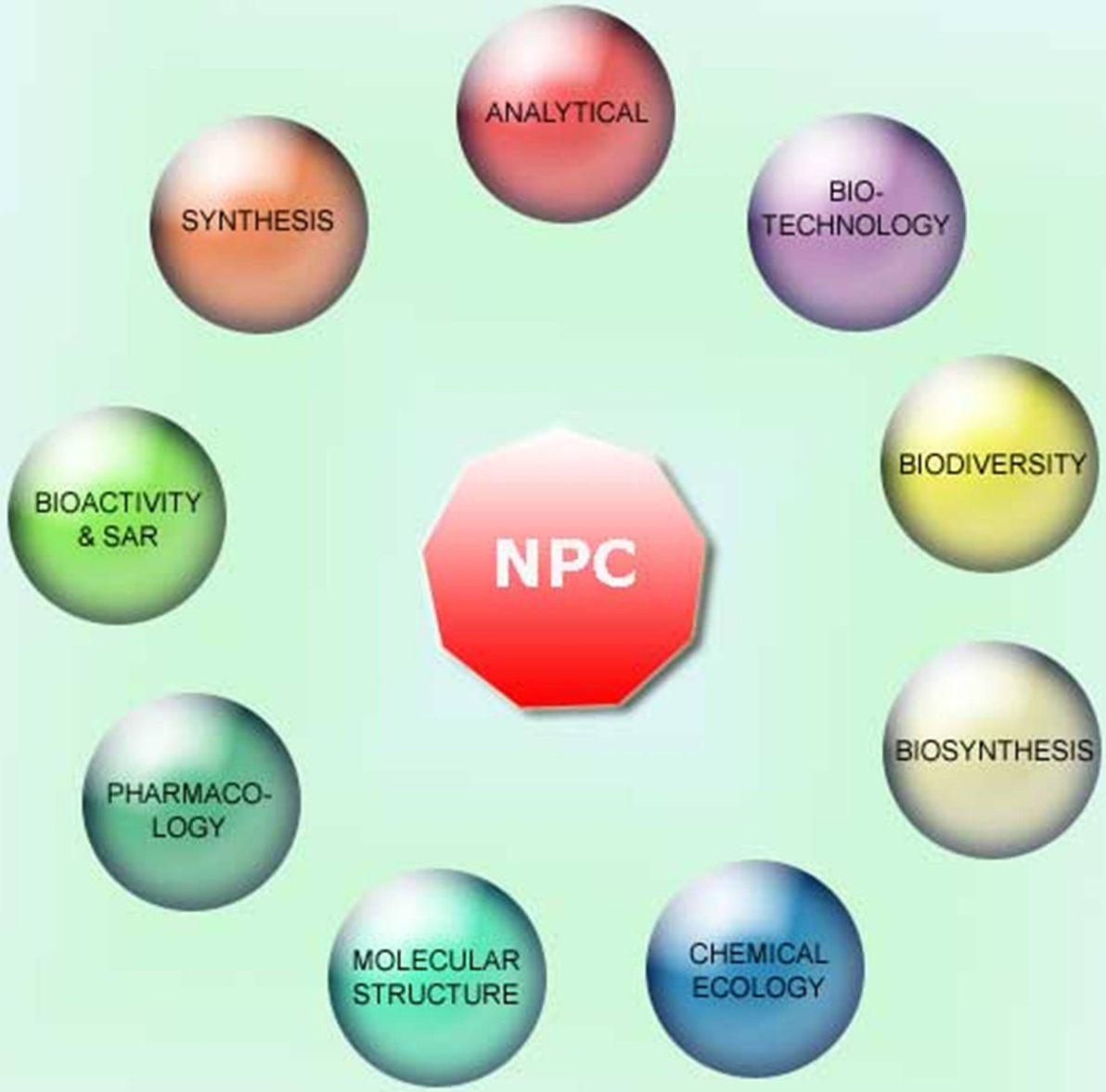

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In Vitro Antiviral Activity of *Heterophyllaea pustulata* Extracts

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The antiviral activity was tested of different polarity extracts, with differing chemical composition, obtained from aerial parts of *Heterophyllaea pustulata* Hook f. (Rubiaceae) against Herpes Simplex Virus Type I (HSV-1) and Saint Louis Encephalitis Virus (SLEV). The Vero cell line was employed as a host cell for the antiviral assessment of benzene (Ben), ethyl acetate (EtOAc) and ethanol (EtOH) extracts by means of the Neutral Red uptake assay and plaque reduction test. None of the extracts showed antiviral activity against SLEV. Only the extracts (Ben and EtOAc) with a high content of anthraquinones (AQs) inhibited HSV-1 replication, exhibiting Selectivity Index (SI) values of 2.7 and 2.4, respectively. Therefore, these extracts could be good candidates as natural sources for antiviral drug development against HSV-1.

Keywords: Antiviral, Antiherpetic activity, Anthraquinone extracts, *Heterophyllaea pustulata*, Cytotoxicity, Selectivity index.

Heterophyllaea pustulata Hook f. (Rubiaceae) is a shrub that grows in the Andean region of northwestern Argentina and Bolivia, popularly known as "cegadera", "ciegadera" o "saruera" [1]. We previously demonstrated the *in vitro* antibacterial and antifungal activity of different extracts of *H. pustulata*, as well as their low *in vivo* toxicity [2]. Chemical investigation of these bioactive extracts showed that they contain several metabolites (anthraquinones, flavonoids and asperuloside), with a predominance of 9,10-anthraquinone aglycones (AQs): soranjidiol, soranjidiol 1-methyl ether, rubiadin, rubiadin 1-methyl ether, damnacanthal, damnacanthol, 2-hydroxy-3-methyl anthraquinone, heterophylline, pustuline and 5,5'-bisoranjidiol [2,3]. Some of these exhibit particularly interesting *in vitro* activities, such as antimicrobial, antitumor, antileukemic, antifungal, and antiparasitic properties, and even antiviral effects against Human Immunodeficiency Virus (HIV) [4-9].

Continuing with the study of *in vitro* biological activities of the extracts obtained from this plant species, in the present work we assessed the *in vitro* antiviral activity of different polarity extracts and hence, with differing chemical composition, against two viral models: Herpes Simplex Virus Type-1 (HSV-1, DNA virus) and Saint Louis Encephalitis Virus (RNA virus).

Herpes Simplex infections affect a high proportion of the entire world population, with recurring episodes that can lead to more serious disorders (for example, keratoconjunctivitis, encephalitis, and meningitis) [10]. Sometimes, these do not respond to the usual antiviral therapy (acyclovir and its derivatives) because the HSV develops resistance to them [10-14]. On the other hand, the diseases caused by arbovirus, such as SLEV (flavivirus, Flaviviridae), belong to pathologies considered a health problem with high economic impact in South America. They should thus be categorized under the term ''neglected tropical disease'' for having no proper therapeutics [15]. It is, therefore, necessary to find new antiviral agents; in this search, we adhere to the guidelines and advice promoted by the World Health Organization (WHO) in relation to native plant-based research to obtain new medicinal agents [16,17].

Chemical composition: As indicated in Table 1, extracts from the aerial parts of *H. pustulata* have either different kinds of secondary metabolites or are found in different ratios. Ben and EtOAc extracts possess the same AQ derivatives, but the amount of them is higher in the Ben extract. Moreover, soranjidiol, rubiadin, rubiadin 1-methyl ether and 5,5'-bisoranjidiol were detected at trace levels in the EtOH extract (< 0.05% P/P, based on dried extract). Flavonoids and asperuloside were found in the EtOAc and EtOH extracts, where 3-*O*-β-D-(6"-acetylglucosyl) quercetin was identified as the main flavonoid, followed in a decreasing order by isoquercitrin and quercetin (Table 1); asperuloside was found at a higher percentage in the EtOH extract.

Table 1: Chemical composition of extracts obtained from aerial parts of *H. pustulata*.

Secondary metabolites	%P/P (g of compound in 100 g of dried extract)[a]		
AQs	**Ben**	**EtOAc**	**EtOH**
soranjidiol	1.80 ± 0.04	0.30 ± 0.02	tl[b]
soranjidiol 1-methyl ether	0.28 ± 0.02	0.20 ± 0.02	-[c]
rubiadin	1.44 ± 0.04	0.23 ± 0.02	tl[b]
rubiadin 1-methyl ether	0.67 ± 0.04	0.17 ± 0.02	tl[b]
damnacanthal	0.27 ± 0.02	0.08 ± 0.02	-[c]
damnacanthol	0.57 ± 0.02	0.06 ± 0.02	-[c]
2-OH-3-CH_3 AQ	tl[b]	tl[b]	-[c]
heterophylline	0.39 ± 0.02	0.35 ± 0.02	-[c]
pustuline	0.13 ± 0.02	0.08 ± 0.02	-[c]
5,5'-bisoranjidiol	0.22 ± 0.02	0.32 ± 0.02	tl[b]
Flavonoids			
quercetin	-[c]	0.09 ± 0.02	tl[b]
isoquercitrin	-[c]	0.11 ± 0.02	1.18 ± 0.02
3-*O*-β-D-(6"-acetylglucosyl) quercetin	-[c]	0.19 ± 0.02	2.25 ± 0.02
Iridoid			
asperuloside	-[c]	0.06 ± 0.02	0.09 ± 0.02

[a]See References [2,3,18]; [b]tl (trace level) < 0.05 %P/P; [c](-) absent.

Cytotoxic activity: Table 2 depicts the Maximum Non Cytotoxic Concentration (MNCC), subtoxic concentration (SubTC) and the concentration that reduces the viable cells to 50% (CC_{50}) of each extract. From the analysis of the CC_{50} values, we determined that the EtOH extract was less cytotoxic than the others. Although the Ben extract would be the most cytotoxic in terms to its CC_{50} value, it generated less cytopathic effect (CPE) at low concentrations. Therefore, the MNCC could be calculated on the basis of its CPE and dose-response curve (Figure 1), which has allowed establishing a concentration range of 1 to 70 µg/mL (MNCC), approximately, where cellular viability (CV) is greater than 90%, with no evidence of cell damage. Even for this extract, we determined a SubTC value higher than those estimated for the EtOAc and EtOH extracts (Table 2). By means of this, we observed that the Ben extract exhibits a concentration range wider than those of the others, where the CV is greater than 80%. The MNCC for the EtOAc and EtOH extracts could not be determined as none of the concentrations tested yielded a CV greater than or equal to 90% (Figure 1).

Table 2: MNCC, SubTC and CC_{50} values for each extract obtained from aerial parts of *H. pustulata.*

Extracts	MNCC (µg/mL)	SubTC (µg/mL)	CC_{50} (µg/mL)
Ben	69.9 ± 1.5	121.3 ± 0.9	205.5 ± 9.0
EtOAc	-[a]	41.2 ± 1.5	239.5 ± 9.5
EtOH	-[a]	111.2 ± 1.5	288.2 ± 9.4

[a](-) not observed

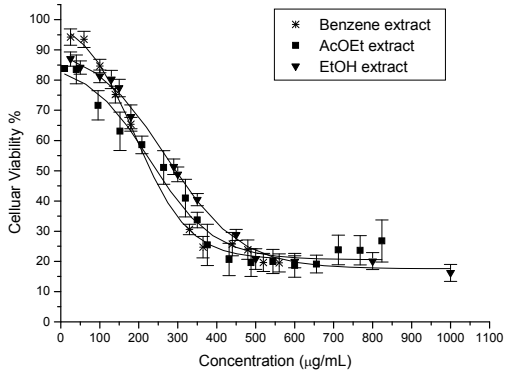

Figure 1: Cellular viability percentage vs. concentration of each extract on Vero cells evaluated by NR uptake assay.

***In vitro* antiviral activity:** No extract tested (Ben, EtOAc and EtOH) showed antiviral activity against SLEV in either of the two methods used (NR uptake and plaque reduction assays). Nevertheless, two of the three extracts (Ben and EtOAc) were active against HSV-1 (Figure 2). The Ben extract inhibited HSV-1 by 47.7 ± 0.6% at the MNCC (Table 3), showing a significant inhibition percentage (%I = 74.2 ± 0.2) at the SubTC, and an increase in inhibitory effect at the CC_{50}. For the EtOAc extract, the highest %I (77.2 ± 0.6) was achieved only at CC_{50} and it was not effective at a SubTC (22.4 ± 0.2 %I) (Table 3). The concentration-%I curves obtained by means of the plaque reduction method (data not shown) were similar to those obtained by the NR uptake assay (Figure 2).

Table 4 shows the concentration needed to achieved 50% of virus inhibition (IC_{50}) and the selectivity index values ($SI = CC_{50}/IC_{50}$) of the extracts tested, as determined by the two methodologies. For those active extracts against HSV-1 (Ben and AcOEt), SI values were greater than 2. Since the EtOH extract was not active against HSV-1 at the concentrations tested, neither its SI value nor the IC_{50} could be determined.

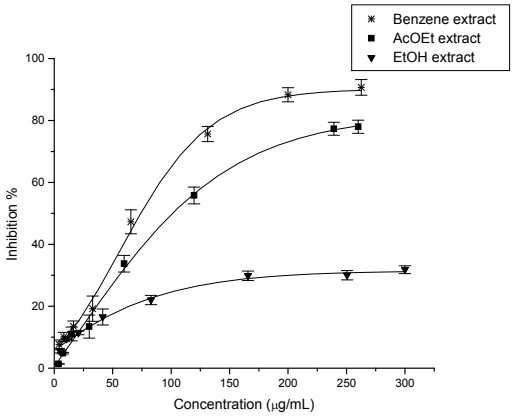

Figure 2: Viral inhibition percentage against HSV-1 vs. concentration of each extract by means of NR uptake assay.

Table 3: Inhibition percentage (%I) against HSV-1 at different concentrations of each extract, obtained from dose-response curves by means of NR uptake assay.

Concentrations (µg/mL)	%I		
	Ben	**EtOAc**	**EtOH**
MNCC	47.7 ± 0.6	nd[a]	nd[a]
SubTC	74.2 ± 0.2	22.4 ± 0.2	25.6 ± 0.1
CC_{50}	88.3 ± 0.3	77.2 ± 0.6	31.0 ± 0.1

[a]nd: not determined

Table 4: IC_{50} and SI values for each extract against HSV-1, determined by two methodologies.

		Plaque reduction	
Extracts	**CC_{50}** (µg/mL)	**IC_{50}** (µg/mL)	**SI**
Ben	205.5 ± 9.0	79.2 ± 2.8	2.6 ± 0.2
EtOAc	239.5 ± 9.5	111.3 ± 8.4	2.2 ± 0.2
EtOH	288.2 ± 9.4	-[a]	nc[b]
		NR uptake	
Extracts	**CC_{50}** (µg/mL)	**IC_{50}** (µg/mL)	**SI**
Ben	205.5 ± 9.0	74.6 ± 3.7	2.7 ± 0.3
EtOAc	239.5 ± 9.5	101.1 ± 9.3	2.4 ± 0.3
EtOH	288.2 ± 9.4	-[a]	nc[b]

[a](-) not observed; [b]nc: not calculated.

The SI is a parameter which measures the relationship between the concentrations of a substance generating antiviral effects and those producing an eventual toxicity on host cells. When the SI value is high, the toxicity to the host cells is low, but the effect against the virus is high. As the extracts of natural products comprise a complex mixture of chemical compounds that would contribute differently to their biological effects, their SI values are normally not particularly high [19,20].There is no cutoff value for this index that could define whether a substance is a good antiviral agent; however, some authors consider that natural products (extracts or compounds) with a SI ≥ 2 would be good antiviral agents [21]. Therefore, the Ben and EtOAc extracts could be a source of potential antiviral agents (Table 4). The results obtained suggest that the antiviral activity exhibited by these extracts could be mainly attributed to their AQs content, since it is precisely in these extracts where such metabolites depict a higher %I (Table 1), some of which have shown antiviral activity against other viral models, such as damnacanthal (moderate activity against HIV) [5]. This affirmation is confirmed by analyzing the composition of the EtOH extract and its bioactivity, since AQ derivatives were detected only at a trace level, and flavonoids and asperuloside were the main components (Table 1). In addition, this extract was inactive against both viruses studied at the concentration ranges tested, despite being the less cytotoxic extract based on its CC_{50} value (Tables 2 and 3).

In summary, the extracts obtained from aerial parts of *Heterophyllaea pustulata* enriched in AQs, and probably the anthraquinone derivatives detected in them, could be good candidates as natural sources for antiviral drug development against HSV-1. Our study is the first report on the antiviral activities of *H. pustulata* extracts against HSV-1, which is important because the extracts can be used to develop herbal medicines. In addition, it should be noted that there are few studies about the antiviral activity of anthraquinones produced through the shikimate-mevalonate biosynthetic pathway, such as the derivatives found in *H. pustulata*. In contrast, the acetate-malonate derived class of anthraquinones has been extensively evaluated for its antiviral effects [22]. Therefore, research is still underway in our laboratory to determinate the cytotoxicity and antiviral activity of each AQ isolated from *H. pustulata* against this virus.

Experimental

Plant material and preparation of extracts: A voucher specimen of *Heterophyllaea pustulata*, collected in La Almona (Jujuy province, Argentina), was deposited at the Botanical Museum of Córdoba (National University of Córdoba) as M.E. Lázzaro s/n, CORD 305. The dried aerial parts (387 g) were crushed and then successively extracted in a Soxhlet apparatus with increasing polarity solvents. Defatting with *n*-hexane was followed by benzene (Ben), ethyl acetate (EtOAc) and ethanol (EtOH), through which the 3 extracts tested in this study were obtained: Ben (5.04 g), EtOAc (15.18 g) and EtOH (26.48 g). The extracts used in this research were previously studied chemically, allowing the identification and quantification of AQs, flavonoids and asperuloside [2,3,18].

Reagents: We used the following reagents: Eagle's minimum essential medium (MEM) (Gibco), Fetal calf serum (FCS) (Natocor), L-glutamine (Calbiochem), gentamicin (Klonal), dimethyl sufoxide (DMSO) (Tetrahedron), Neutral Red (NR) (Gibco), and phosphate buffer saline (PBS). The solvents were distilled before use.

Cells and virus: African green monkey kidney cells (*Cercophitecus aethiops*, Vero 76 ATCC CRL-587) were used as host cells. They were grown and kept alive under humid atmosphere with 5% CO_2 at 37°C. Whereas MEM supplemented with 10% FCS, 1% L-glutamine and gentamicin (50 µg/mL) was used as growth medium, MEM plus 2% FCS containing the same formulation as that described above was used as a maintenance medium (MM).

In order to determine the antiviral activity of the extracts, Herpes Simplex Virus Type-1 (HSV-1) strain Kos, as representative of DNA viruses and Saint Louis Encephalitis Virus (SLEV) strain 78V6508, as representative of RNA viruses, were used. These viruses have been completely characterized and they are widely used in research and diagnosis at the Institute of Virology, School of Medicine, National University of Córdoba. Virus stocks were propagated and titrated according to the methods described by Contigiani and Sabatini [23].

Samples for biological assays: Stock solutions of each extract (100 mg/mL) were prepared in PBS with 1% DMSO as co-solvent, and serial dilution was carried out with MM containing 1% DMSO in order to avoid precipitation of extracts. Controls were prepared to ensure the same percentage of DMSO (1%) in the MM.

In vitro cytotoxicity assay: Alterations produced by each extract on the morphology of Vero cells were observed by optical microscopy to assess cytopathic effect (CPE) [24]. From a stock solution of each extract, consecutive 2-fold serial dilutions were performed, within a range of 1 to 1000 µg/mL according to the solubility of each extract. Each dilution was added in duplicate to a confluent cell monolayer ($2.0 \pm 0.6 \times 10^5$ cells/mL incubated for 48 h to reach at least 95% confluency; these conditions were applied to all assays performed in this work). Cells incubated only with MM were used as cell controls (CC, 2 replicates). The culture plates were incubated at 37°C in 5% CO_2 for 72 h, and the morphology of cells was observed daily for microscopically detectable alterations, e.g. loss of monolayer, rounding, shrinking of the cells, granulation, and vacuolization in the cytoplasm [25].

The cellular viability (CV) was measured by the NR uptake assay performed following a procedure similar to that described by Borenfreund and Puerner [24]. The same 15 dilutions used in the CPE test were added to a confluent monolayer of cells, using 3 replicates of each concentration and CC. The absorbance of the extracted NR after 48 h incubation at 37°C in 5% CO_2 was measured at 540 nm by using a microplate reader (BioTek ELx800). The decrease in CV was calculated as the percentage of the CC (100% viability). The CC_{50} was estimated by GraFit 7.0. MNCC was defined as the maximal sample concentration showing more than 90% viable cells and exerting no cytotoxic effect, as detected by microscopic monitoring [26]. A SubTC was determined for all extracts tested, defined as the concentration that causes 10 - 20% cellular death [27] and produces slight morphological changes observed by microscopy (less than 20% of swollen and rounded cells, with cytoplasmic inclusions, slight vacuolization, and the nuclear membrane remaining intact).

In vitro *antiviral activity*

NR uptake assay: The confluent monolayer of Vero cell was infected with either HSV-1 or SLEV at an MOI of 0.1 - 1. After 1 h incubation for virus adsorption (37°C in 5% CO_2), 6 different concentrations of each extract were added (consecutive 2-fold serial dilutions from CC_{50}), with 4 replicates per dilution. Non-infected untreated cells in MM (cell controls, CC), uninfected and treated cells with the tested concentrations of each extracts (sample controls, SC) and infected untreated cells (virus controls, VC) were included in quadruplicate. The culture plates were incubated under the same temperature and atmospheric conditions for 3 days for HSV-1 and 7 days for SLEV; the assay was then carried out following the procedure described by Borenfreund and Puerner [24, 28]. The RN absorbance was measured in the same way as that in the RN uptake assay. The percentage of inhibition (%I) was calculated as shown by Gescher *et al* [29]; such values (%I) were plotted against the corresponding concentration of each extract.

Plaque reduction assay: The viral suspension (100 pfu/well) was inoculated on a confluent monolayer of Vero cells. After 1 h adsorption, residual inoculums were discarded and the cells were washed before adding the concentrations of each extract previously used in the NR test, with 2 replicates per dilution. Virus and cell controls were included in duplicate. After 30 min incubation, each well was overlaid with semi-solid agarose medium (1.0% agarose in double concentration of MEM). Plaques were counted after 3-7 days of incubation at 37°C, according to the viral model, following the methodology described by Cheng *et al.* [30]. The results were expressed as percentage of plaque inhibition (%I) by comparison with viral controls [29]. These values (%I) were plotted against the corresponding concentration of each extract. The concentration needed to achieve 50% of virus inhibition (IC_{50}) was estimated using both assays. The selectivity index (SI) was measured as the ratio CC_{50}/IC_{50} for each extract [26]. Acyclovir (Fada, lyophilized

powder for intravenous administration) was used as positive control of HSV-1 in both methodologies (20 µg/mL), whereas a positive antiviral control against SLEV was not included because of the lack of effective antiviral drugs for this virus.

Data analysis: MNCC, SubTC, CC_{50} and IC_{50} values were graphically obtained from dose-response curves with a non-lineal regression analysis (Sigmoidal Origin, $R^2 > 0.9$). The values were expressed (mean ± standard error) from 3 different experiments. Thus, for each concentration, 6 replicates were carried out to determine cytopathic effect, 9 to quantify cell viability, 12 to evaluate antiviral activity by NR uptake assay, and 6 to test plaque reduction.

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Review/Account

Natural Product Communications
2012
Volume 7, Number 8

Contents

Continued inside backcover