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# Phytochemical screening and *in vitro* antiherpetic activity of four *Erythroxylum* species

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**SUMMARY. Leaves from four Cuban *Erythroxylum* species were submitted to phytochemical screening. Alkaloids, triterpenoids/steroids, lactones/coumarins, reducing sugars, phenols/tannins and flavonoids were identified as principal secondary metabolites. Hydroalcoholic lyophilized leaf extracts from the four *Erythroxylum* species were assayed for activity against herpes simplex virus type 1. Hydroalcoholic extracts of two species *(E. areolatum* and *E. confusum)* inhibited the growth up of this virus.**

*RESUMEN.* "Análisis fitoquímico y actividad antiherpética *in vitro* de cuatro especies de *Erythroxylum*". Hojas de cuatro especies de *Erythroxylum* que crecen en Cuba fueron sometidas a análisis fitoquímico, identificándose alcaloides, triterpenoides/esteroides, lactonas/cumarinas, azúcares reductores, fenoles/taninos y flavonoides como sus principales metabolitos secundarios. Extractos hidroalcohólicos liofilizados de hojas de las cuatro especies de *Erythroxylum* estudiadas fueron ensayadas con respecto a su actividad sobre el virus del herpes simplex tipo 1. Los extractos hidroalcohólicos de dos de las especies *(E. areolatum* and *E. confusum)* inhibieron el crecimiento del virus.

## INTRODUCTION

Genus *Erythroxylum* (Erythroxylaceae) consists of small tree species distributed in tropical America, Africa and Madagascar. In Cuba, 22 species of *Erythroxylum* have been identified. From these, sixteen species are endemic [1]. There are not many reports of chemical and pharmacological studies in this group. However, it has been used on ethno-medical practices with applications as anti-inflammatory and in the treatment of bronchitis and other respiratory affections [1,2]. Branches and leaves are the principal parts used [3,4]. Alkaloids and flavonoids are the main chemical families described in this genus [5,6]. Aqueous and hydroalcoholic extracts from leaves of plants in this genus have shown an important anti-microbial [7], anti-herpes simplex virus type-I [8] and anti-human immunodeficiency virus (HIV) infection [9] as principal pharmacological activities. Particularly, positive antiviral activity has been reported against the herpes simplex virus (HSV) for *Erythroxylum citrifolium*, *E. lucidum* and *E. laurifolium* extracts with *in vitro* assays [8,10].

From these previous results, a phytochemical and pharmacological screening of leaves from *E. confusum* Britt., *E. areolatum* L., *E. havanense* Jacq. and *E. alaternifolium* A. Rich., *var. alaternifolium* was carried out. The main purpose of the pharmacological study was to evaluate the potential antiviral activity of these natural agents.

## MATERIALS AND METHODS

### *Plant extracts*

Leaves of *Erythroxylum* species mentioned above were collected in Pinar del Río, Cuba, in April 2001 and authenticated by Armando Urquiola, with specimens deposited at the Herbarium of Instituto Pedagógico of Pinar del Río *(E. confusum:* 9191, *E. areolatum:* 9193, *E. havanense:* 9207 and *E. alaternifolium:* 9201).

Samples were dried in the shadow for 20 days. Finally the leaves were triturated separately in a disc mill. For phytochemical screening 10 g of the powdered leaves were weighed and submitted to reflux for an hour with 100 mL of n-hexane. The extract was filtered and the solid debris submitted to another reflux for an hour with 100 mL of ethanol. Then this procedure was repeated with 100 mL of distilled water.

Maceration was carried out on 10 g of veg-

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etable material from each species and mixed with 100 mL of 70% ethanol leaving to macerate for three days, and then decanted. This procedure was repeated three times. The hydroalcoholic extracts were then filtered, the ethanol eliminated and the residue lyophilized. These samples were used for cytotoxicity and antiviral assays.

### *Viruses and cells*

The strain HSV 1 was propagated on VERO (African's monkey green kidney) cells (ATCC Number: CCL-22). VERO cells were grown as monolayers at 37 °C in a humidified 5% $CO_2$ atmosphere using 199 medium (Sigma) supplemented with 5% inactivated calf foetal serum (FCS) (Hyclone), 0.1% L-glutamine and 100 UI/mL neomycin sulphate. The HSV 1 strain used was a kind donation of the Virology Department of the "Pedro Kouri" Tropical Medicine Institute (Cuba), isolated from a patient there.

For virus titration the cells were grown in 96-well tissue culture plates, incubated for one hour at 37 °C with serial 10-fold diluted virus suspension. After adsorption, the inoculum was removed and maintenance culture medium (199, without FCS) was added. Titres was calculated as 50% tissue culture infectious doses ($TCID_{50}$)/mL using the Reed and Muench method [11] to estimate endpoints. All plates were incubated at 37 °C and observed daily for cytopathic effect (CPE). Estimation of the endpoints was made on the 5$^{th}$ day.

### *Phytochemical screening*

Each extract fraction (n-hexane, ethanol and water) was analyzed by specific reactions, as described by Schabra *et al.* [12]. The color intensity of extracts and/or the appearance of solids in them during the identification reactions, allow a semi-quantitative evaluation of the presence of secondary metabolites [13].

### *Cytotoxicity assay*

Cytotoxicity of *Erythroxylum* hydroalcoholic extracts was evaluated using an assay based in the color change which occurred following the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT-Sigma, St. Louis, MO) by mitochondrial enzymes [14], which has been previously used to measured drug cytotoxicity [15].

The assays were performed using 96-well flat bottom tissue culture plates. Increasing concentrations (31.25-1000 µg/mL) of the plant extracts were added to the monolayers of each cell type in triplicate and the plates were incubated at 37 °C in a 5% $CO_2$ atmosphere. After 72 h, MTT was added, the cultures were incubated for three hours and the absorbance at 570 nm was measured using a 96-well plate ELISA reader. The 50% cytotoxic concentration ($CC_{50}$) was then determined using the EXCEL Program (Microsoft® EXCEL 97, 1985-1997, Microsoft Corporation, USA).

To confirm the results obtained with the MTT assay, the monolayers were also observed microscopically for estimating CPE (cytopathogenic effect), i.e. rounding and other marked morphologic changes with respect to control cells.

### *Antiviral activity assay*

Antiviral activity of the hydroalcoholic extracts of *Erythroxylum* species was evaluated *in vitro* by the CPE method using 96-cells flat bottom tissue culture plates. In these assays, 100 µL/well of culture medium containing different concentrations of plant extracts (31.25-1000 µg/mL), were added in triplicate to confluent monolayers of each cell type. After 90 minutes incubation at 37 °C in a 5% $CO_2$ atmosphere incubator the virus control wells exhibited 70-100% CPE. All wells were then observed under a light microscope and scored for viral CPE, and by the MTT method as previously described [16]. Regression analysis was used to calculate the 50% effective concentration (EC50). A Selective Index (SI) was calculated for the *Erythroxylum* extracts against the HSV 1 by dividing the appropriate $CC_{50}$ value by the corresponding $EC_{50}$.

### *Statistical analysis*

Comparisons between the effect produced by different concentrations of the plant extracts and the untreated controls were done by ANOVA followed by Dunett's test. The level of significance was set at $p \leq 0.05$. Means, S.D., $CC_{50}$ and $EC_{50}$ values were calculated using regression analysis.

## RESULTS

Phytochemical screening results of four *Erytrhoxylum* species leaves are shown in Table 1. The main secondary metabolites identified in polar extracts were triterpenoids/steroids, phenols/tannins, lactones/coumarins and reducing sugars. Alkaloids, quinones, saponines and cardiac glycosides were identified in low amounts, and lipids/essential oils and carotenoids were not detected. Amines were only identified in *E. havanense*, and flavonoids were detected in high levels in two, *E. areolatum* and *E. confusum*. In the other two species the presence of flavonoids was very low.

| Assays | Type of extracts | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Hexane | | | | Ethanol | | | | Water | | | |
| | Species | | | | | | | | | | | |
| | I | II | III | IV | I | II | III | IV | I | II | III | IV |
| Flavonoids | NA | NA | NA | NA | +++ | ++ | – | – | +++ | +++ | – | + |
| Phenols/tannins | NA | NA | NA | NA | +++ | +++ | ++ | +++ | +++ | +++ | +++ | ++ |
| Alkaloids | – | – | – | – | – | – | – | – | – | – | – | – |
| Reducing sugars | NA | NA | NA | NA | +++ | +++ | ++ | +++ | +++ | ++ | + | ++ |
| Lactones/coumarins | – | – | – | – | +++ | +++ | + | +++ | NA | NA | NA | NA |
| Triterpenoids/steroids | +++ | ++ | + | ++ | ++ | + | +++ | + | NA | NA | NA | NA |
| Cardiac Glycosides | NA | NA | NA | NA | – | ++ | – | – | NA | NA | NA | NA |
| Quinones | – | – | – | – | + | – | – | – | NA | NA | NA | NA |
| Lipids/essential oils | – | – | – | – | NA | NA | NA | NA | NA | NA | NA | NA |
| Carotenoids | – | – | – | – | NA | NA | NA | NA | NA | NA | NA | NA |
| Saponins | NA | NA | NA | NA | – | – | – | – | – | + | – | ++ |
| Amines | NA | NA | NA | NA | – | – | +++ | – | NA | NA | NA | NA |
| Mucilages | NA | NA | NA | NA | NA | NA | NA | NA | – | – | – | – |

**Table 1**. Four *Erythroxylum* species leaves phytochemistry screening results. Positive assay (+), negative assay (-), not assayed (NA). **I** = *Erythoxylum areolatum* Linn.; **II** = *E. confusum* Britt.; **III** = *E. havanense* Jacq.; **IV** = *E. alaternifolium* A. Rich. var. *alaternifolium*.

| Species | $CC_{50}$ (µg/mL) ± SD | $EC_{50}$ (µg/mL) ± SD | SI ($CC_{50}$/ $EC_{50}$) |
|---|---|---|---|
| *E. areolatum* Linn. | > 1000 ± 11.5 | < 31.25 ± 12.3 | > 32 |
| *E. confusum* Britt. | > 1000 ± 9.2 | < 31.25 ± 11.1 | > 32 |
| *E. havanense* Jacq. | > 500 ± 7.4 | – | – |
| *E. alaternifolium* A. Rich | 125 ± 10.5 | 125 ± 9.3 | 1 |

**Table 2**. *In vitro* cytotoxicity and antiviral activity of hydroalcoholic extracts from *Erythroxylum* species leaves against HSV 1. **$CC_{50}$**: medium cytotoxicity concentration, **$EC_{50}$**: medium effective concentration, **SI**: Selective Index, **SD**: Standard Deviation.

Cytotoxic effects and *in vitro* anti-HSV 1 activity evaluation of hydroalcoholic extracts of these four *Erythroxylum* species was carried out. Results are shown in Table 2.

To assay for cytotoxic effects of the *Erythroxylum* hydroalcoholic extracts on VERO cells used to propagate the HVS-1, they were incubated with increasing amounts (from 31.25 to 1000 µg/mL) of the hydroalcoholic plant extract. The viability of the treated cultured cultures was investigated using the MTT method. The results indicated that extract concentrations up to 500 µg/mL *(E. areolatum)*, 250 µg/mL *(E. confusum)*, 500 µg/mL *(E. havanense)* and 125 µg/mL *(E. alaternifolium)* did not impair the cell viability at all with respect to the corresponding untreated cultures ($p < 0.05$). In all cases the toxic effects observed at higher *Erythroxylum* extract concentrations consisted of the presence of granules and vacuoles that increased in size and number with the plant extract concentration. The $CC_{50}$ of *Erythroxylum* extracts were calculated in the range mentioned above.

The *E. areolatum* and *E. confusum* extracts exhibited significant antiviral activity on the HSV 1 with mean $EC_{50}$ values of 31.25 µg/mL and average SI of 32, for both (Table 2). In contrast, no effect of the plant extracts was detected using the same protocol for *E. havanense* and *E. alaternifolium* species. The $EC_{50}$ value of *E. havanense* extract was out the range of the $CC_{50}$ values and the $EC_{50}$ value of *E. alaternifolium* extract was the same to its respective $CC_{50}$ value.

## DISCUSSION

As it can be observed in Table 1, most of the secondary metabolites were identified in the polar (ethanol and water) extracts. The concentration of polar metabolites is higher than non-polar metabolites in leaves of these species. Alkaloids are one of the characteristic secondary metabolites in leaves, stems and fruits of this

genus (tropane alkaloids) [17]. They were only identified in aqueous extracts of *E. havanense* and *E. alaternifolium*, in very low amounts. This result could be a consequence of the collection season [18].

Flavonoids are usually used as chemotaxonomic markers for *Erythroxylum* species [19,20]. For these species, flavonoids were detected in high amounts in *E. areolatum* and *E. confusum*, whereas flavonoid levels in *E. alaternifolium* were low and not detected in *E. havanense*. *E. alaternifolium* and *E. havanense* species showed marked differences in the content of amines, alkaloids and flavonoids.

The hydroalcoholic extracts from *E. areolatum* and *E. confusum* exhibited selective antiviral activity in tissue culture (SI ≥ 10) against HSV 1 following published criteria [21]. However, no antiviral activity was observed for the other two *Erythroxylum* species studied.

The negative results against HSV 1 for *E. alaternifolium* and *E. havanense* could be due to the lower amounts of flavonoids [3,7] (Table 1), as their antiviral activity against a number of virus is widely known [22].

A pharmacological study of a *E. laurifolium* hydroalcoholic extract using HSV 1 demonstrated inhibition to high concentrations of virus growth, and outlines the possible antiviral effect of the tannins in this extract [10]. Tannins isolated from other genus have been reported as antiviral agents [21]. Tannin presence was high for all *Erythroxylum* species (Table 1), so in this case the tannins do not seem to have the main responsibility in the antiherpetic activity.

The $CC_{50}$ and $EC_{50}$ values obtained by the MTT method show a good correlation with the results found after microscopic observation of the CPE, as reported for an *Euphorbiaceae* derived compound [20].

The isolation and purification of secondary metabolites from leaves of *E. areolatum* and *E. confusum* collected in April/2001 guided by antiviral bioassays will be the continuation of this preliminary study.

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