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Preliminary Phytochemical Screening and *in vitro* Antiherpetic activity of *Erythrina fusca* Lour.

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SUMMARY. Sixteen species of the genus *Erythrina* (Papilionaceae) have been described in Cuba, four of them as endemic. From these different species of *Erythrina* significant analgesic, diuretic, sedative and antiviral properties were observed. An initial collection was carried out in September/2000 and a second one in April/2001. The preliminary phytochemical screening of branches, leaves (first collection) and bark (both collections) of *Erythrina fusca* yielded alkaloids, flavonoids, triterpenoids, steroids, saponins, lactones, coumarines, reducing sugars, carotenoids, amines and cardiac glycosides. Polar extracts were assayed for activity against herpes simplex virus types 1 and 2. These extracts inhibited the growth of herpes simplex virus type 1 at an effective medium concentration of 243 µg/mL and herpes simplex virus type 2 at 109.5 µg/mL.

RESUMEN. "Revisión Fitoquímica Preliminar de la Actividad Antiherpética *in vitro* de *Erythrina fusca* Lour." Dieciséis especies pertenecientes al género *Erythrina* (Paplilianceae) han sido descritas para Cuba, cuatro de ellas endémicas. Se ha mencionado que estas especies poseen significativa actividad analgésica, diurética, sedante y antiviral. Una recolección original se realizó en septiembre de 2000 y una segunda en abril de 2001. El análisis fitoquímico preliminar de ramas, hojas (primera recolección) y corteza (ambas recolecciones) de *Erythrina fusca* reveló la presencia de alcaloides, flavonoides, triterpenoides, esteroides, saponinas, lactonas, cumarinas, azúcares reductores, carotenoides, aminas y glicósidos cardíacos. La actividad de los extractos polares fue ensayada contra los virus del herpes simplex del tipo 1 y 2. Estos extractos inhibieron el crecimiento del virus del herpes simplex tipo 1 a una concentración media efectiva de 243 µg/mL y al virus del herpes simplex tipo 2 a una concentración media efectiva de 109,5 µg/mL.

INTRODUCTION

One hundred and fifteen *Erythrina* species (Papilionaceae) have been described. They grow throughout America. The greatest concentration of *Erythrina* species is found in Southern Mexico and Central America. This genus has a wide range of morphological variation and ecological diversity [1]. In Cuba sixteen *Erythrina* species have been described. Four of them are endemic species: *Erythrina elenae* Howard, *E. grisebachii* Urb., *E. acunae* Borhidi and *E. cubensis* C. Wr. These species have a specific distribution in the Cuban archipelago [2].

Locally the *Erythrina* species is known as "Coral tree" and is frequently cultivated as an ornamental plant. The Coral tree has different uses, as forage, as a support for valuable climbing crops and as a shade tree for coffee, cacao and other crops [1]. Several Central American *Erythrina* species have edible flowers that are utilized as food. *E. americana* is an example of this, and is used in some places of Mexico [3]

Different parts of *Erythrina* species have been used in traditional medicine in the treatment of some pathologies due to their analgesic, diuretic, sedative or antiviral properties [4]. Alkaloids, flavonoids, pterocarpans and lectins are characteristic of the *Erythrina* genus. Alkaloids have been reported in extracts of *Erythrina* spp. as responsible of anti-inflammatory activity [5]. Flavonoids and pterocarpans have been isolated from *E. glauca* Willd. and *E. lysistemon*

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Hutch. These compounds inhibited the cytopathic effects of *in vitro* Human Immunodeficiency Virus type 1 infection in a human T-lymphoblastoid cell line [6,7]. Another type of flavonoids (isoflavones and flavanones) has been reported with antibacterial and antifungal properties [8,9]. Lectins isolated from *E. cristagalli* L. and *E. Corallodendron* Griseb. have been assayed in human gastrointestinal neoplasm. The anticarcinogenic activity of these compounds was demonstrated by Baldus [10].

The existence of viruses responsible for sexually transmitted diseases, such as Human Papiloma Virus, Human Immunodeficiency Virus, Hepatitis B and herpes simplex virus type 2 (HSV-2) has affected millions of people around the world. The ethnomedical practice has supplied an alternative approach for the treatment of these diseases, for example hepatitis and herpes virus [11]. For this reason the search for antiviral agents obtained from plants has become a systematic and important practice in the field of the natural products.

Herpes simplex virus type 1 (HSV-1) provokes feverish blisters. The lesions appear around the lips, mouth and pharyngeal mucus including nose, mouth, face and ears. This virus has been isolated from the facial nerves. There are not curative treatments. Topical medicines can be applied to relieve the pain, stinging and inflammation [12].

Genital herpes infection is a life-long disease and may result in painful and recurrent genital lesions, systemic complications, serious psychosocial morbidity, and rare but serious outcomes in neonates born to infected women. Permanent neurological handicap and death may accompany it. Genital HSV transmission is usually due to asymptomatic viral shedding by people who are unaware that they are infected. Clinical screening fails to detect most infections. The prevalence of HSV genital infections increases with age and number of sexual partners, with higher rates in specific ethnic and low socioeconomic groups. However, infection is not restricted to high-risk populations [13].

Literature reports that ethanol extracts of stems *E. abyssinica* assayed for activity against herpes virus gave positive results [14]. These results stimulated our interest to search for antiviral alternatives in *E. fusca* Lour.

MATERIALS AND METHODS

Plant material

Erythrina fusca (Papilionaceae) specimens were authenticated by Adelaida Barreto, at "Centro de Investigaciones del Medio Ambiente", Camagüey, Cuba. Samples were collected in San Juan y Martínez, Pinar del Río, Cuba. Collections were carried out in two different seasons in successive years, September/2000 and April/2001. Voucher specimens (HPPR-9195, HPPR-9196, respectively) were deposited at the "Instituto Pedagógico" of Pinar del Río. Branches, leaves and bark were dried in the shadow for 20 days. The samples were ground in a disc mill.

Preparation of the extracts

For phytochemical screening, 10 g of each vegetable material were successively submitted to reflux extraction for one hour with 100 mL of n-hexane, ethanol and distilled water. Each extract was analyzed by specific reactions, as described by Schabra *et al.* [15].

For cytotoxicity and antiviral assays, the samples were obtained by extraction with water (infusion and decoction) and ethanol 70% (maceration).

Decoctions were prepared from 10 g of leaves, branches or bark. They were heated separately at 100 °C in 100 mL of distilled water for twenty minutes. The extract was filtered and lyophilized.

Infusions were prepared from another 10 g of each part of the plant. These vegetal materials were macerated separately with 100 mL of water at boiling temperature for twenty minutes. The extract was filtered and lyophilized.

Hydroalcoholic extracts were obtained from 10 g of vegetable material from each species and added with 100 mL of 70% ethanol leaving to macerate for three days, and then decanted. This procedure was repeated three times. The hydroalcoholic extract was then filtered, ethanol eliminated and the residue lyophilized. The lyophilized material was stored at 2 °C until used for the assays.

Cells and viruses

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, 100 UI/mL neomycin sulphate. The HSV-1 strain used was isolated from a patient at the "Instituto of Medicina tropical, Pedro Kouri ", Cuba. The Institute's Virology Department gently donated the strain.

For virus titration, cells grown in 96-well tis-

sue culture plates were incubated for 1 h 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 [16] 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 5th day.

Phytochemical screening

Each extract fraction (n-hexane, ethanol and water) was analyzed by specific reactions, as described by Schabra *et al.* [15]. The color intensity of extracts and/or the appearance of solids in them during the identification reactions allow establishing a semi-quantitative presence of the secondary metabolites [17].

Cytotoxicity assay

The cytotoxicity of the *Erythrina* 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 [18], which has been previously used to measured drug cytotoxicity [19].

The assays were performed using 96-well flat bottom tissue culture plates. Increasing concentrations (31.25-1000 µg/mL) of the plant extract 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 3 h 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 (citopathogenic effect), i.e. rounding and other marked morphologic changes with respect to control cells.

Antiviral activity assay

The antiviral activity of the *Erythrina* extracts 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 min of incubation at 37 °C in a 5% CO_2 atmosphere incubator virus control wells exhibited 70-100% CPE. All wells were then observed and scored for viral CPE under a light microscope and by the MTT method as previously described [20]. Regression analysis was used to calculate the 50% effective concentration (EC_{50}). A Selective Index (SI) was calculated for the *Erythrina* 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

A preliminary phytochemical screening of branches, leaves (first collection) and bark (both collections) of *E. fusca* yielded alkaloids, lactones/coumarines, saponins, reducing sugars and cardiac glycosides in all parts of the plant. However, triterpenoids/steroids, carotenoids, amines and flavonoids were not detected in all studied parts of the plant (Tables 1 and 2).

Triperpenes/steroids were generally identified in the non-polar extracts of branches, leaves and bark collected in September/2000. These metabolites were not detected in bark collected in April/2001. Carotenoids were only identified in the leaves of the plant.

Amines were detected with high amount in branches and bark collected in September/2000. However, these metabolites were not identified in bark collected in April/2001. Alkaloids were only identified in aqueous extracts of the plant. These compounds were detected with high amount in leaves and branches of this species.

Antiviral and cytotoxicity activity

Cytotoxic effects and *in-vitro* anti HSV-1 and HSV-2 activity evaluation of aqueous and hydroalcoholic extracts of *E. fusca* was carried out (Table 3). To assay for cytotoxic effects of the *Erythrina* extracts on VERO cells used to propagate the HVS-1 and HSV-2, cells were incubated with increasing amounts (from 31.25 to 1000 µg/mL) of plant extracts. The viability of the treated cultured cultures was investigated using

Assay	*Erythrina fusca* Lour. (branches, first collection)			*Erythrina fusca* Lour. (leaves, first collection)		
	hexane extract	ethanol extract	aqueous extract	hexane extract	ethanol extract	aqueous extract
Alkaloids	-	-	+++	-	-	+++
Triterpenoids/steroids	++	-	NA	+++	+++	NA
Quinones	-	-	NA	-	-	NA
Lactones/coumarins	+	++	NA	+	+++	NA
Lipids/essential oils	-	NA	NA	-	NA	NA
Carotenoids	-	NA	NA	++	NA	NA
Saponins	NA	-	+++	NA	+++	+++
Phenols/tannins	NA	-	-	NA	-	-
Amines	NA	+++	NA	NA	-	NA
Reducing sugars	NA	+++	+	NA	+++	+++
Flavonoids	NA	-	-	NA	++	-
Cardiac glycosides	NA	++	NA	NA	+	NA
Mucilage	NA	NA	-	NA	NA	-

Table 1. Phytochemical screening results of *Erythrina fusca* Lour. (branches and leaves, First collection).Positive assay (+), Negative assay (-), Not assayed (NA).

Assays	*Erythrina fusca* Lour. (bark, first collection)			*Erythrina fusca* Lour. (bark, second collection)		
	hexane extract	ethanol extract	aqueous extract	hexane extract	ethanol extract	aqueous extract
Alkaloids	-	-	+++	-	-	+
Triterpenoids/steroids	+	-	NA	-	-	NA
Quinones	-	-	NA	-	-	NA
Lactones/coumarins	+	++	NA	+++	+++	NA
Lipids/essential oils	-	NA	NA	-	NA	NA
Carotenoids	-	NA	NA	-	NA	NA
Saponins	NA	-	+++	NA	-	+++
Phenols/tannins	NA	-	-	NA	-	-
Amines	NA	++	NA	NA	-	NA
Reducing sugars	NA	++	+++	NA	+++	+++
Flavonoids	NA	-	+++	NA	-	+++
Cardiac glycosides	NA	+	NA	NA	+++	NA
Mucilage	NA	NA	-	NA	NA	-

Table 2. Phytochemical screening results of *Erythrina fusca* Lour. (bark of two collections). Positive assay (+), Negative assay (-), Not assayed (Na).

the MTT method. The results indicated that only the decoction of bark, collected in September/2000, inhibited the growth up of HSV type 1 and 2.

Half cytotoxicity concentration (CC_{50}) for the active extract was >8 µg/mL. This extract exhibited significant antiviral activity on the HSV-1 and HSV-2 with mean EC_{50} values of 243 ± 10.9 µg/mL and 109.5 ± 12.5 µg/mL respectively. The average SI was >33 and >73 for HSV-1 and HSV-

Part of the plant	Type of extract	CC_{100} µg/mL	CC_{50} ± SD mg/mL	EC_{50} ± SD HSV-1 µg/mL	SI HSV-1	EC_{50} ± SD µg/mL HSV-2	SI HSV-2
leaves*	D	500					
leaves*	I	500					
leaves*	M	62.5					
branches*	D	500					
branches*	I	500					
branches*	M	250					
bark*	D	> 500	> 8 ± 11,2	243.0 ± 10.9	> 33	109.5 ± 12.5	> 73
bark*	I	500					
bark*	M	125					
bark**	D	> 500					
bark**	M	125					

Table 3. Pharmacological results of *Erythrina fusca*, Lour. collected in September/2000 and April/2001. **I**: Infusion, **D**: Decoction, **M**: Maceration. *: First collection (september/2000), **: Second collection (April/2001). CC_{100}: total cytotoxicity concentration, CC_{50}: medium cytotoxicity concentration EC_{50}: medium effective concentration, **SI**: Selective Index, **SD**: Standard Deviation.

2, respectively as shown in Table 3. In contrast, no effects of the plant extracts were detected using the same protocol for other parts of the plants or other types of extracts.

DISCUSSION

Phytochemical Screening

Erysovine, erythraline, erysothrine, erysothramidine and other alkaloids have been isolated from *Erythrina* species such as *E. latissima* [21]. In good agreement, they gave positive test in all screened species, but they could be present as glycosides or quaternary salts, as they were only detected in aqueous extracts of *E. fusca*. These results are shown in Tables 1 and 2.

Flavonoids are phenolic structures and are frequently present as glycosides in the leaves. Tables 1 and 2 show these secondary metabolites to be localized in leaves and barks of both collections (september/2000 and april/2001). The presence of flavonoids and pterocarpans is common in species of the *Erythrina* genus. Two isoflavanoids, 5-deoxyglyasperin F and 2´-hydroxyneobavaisoflavanone, present in *E. lysistemon*, have been reported as a HIV inhibitory, with EC_{50} of 11.5 and 7.6 µg/mL, respectively [6]. Two pterocarpans, 3-O-methylcalopocarpin and sandwicensin, have also been reported in *E. glauca*, showing activity against HIV with EC_{50} de 0.2 and 2 µg/mL, and a cellular protection of 80-95% and 50-60 %, respectively [6].

Cardiac glycosides are detected in all parts of the plant. Reports about the presence of these structures in species of the genus *Erythrina* have not been found in the literature.

Alkaloids, flavonoids, cardiac glycosides, reducing sugars, saponins and lactonic compounds were identified in bark from both collections (Table 2). The concentrations of alkaloids, lactonic compounds and cardiac glycosides were different for each tissue. The intense coloration and the solid amount during the identification reactions indicated the different concentrations of these metabolites. Triterpenoids, steroids and amines were only identified in the bark of first collection.

Antiviral and cytotoxicity activity

In vitro antiviral activity against HSV type 1 and 2 was evaluated for the extracts obtained by decoction, infusion and ethanol 70% maceration of three parts of the plant: leaves, branches and bark. Table 3 shows the most important inhibition of the HSV-1 and HSV-2 was only achieved by the total extract obtained by decoction from bark. For this reason, a preliminary phytochemical screening and antiviral evaluation of bark *E. fusca* collected in Abril/2001 was carried out. The results of this study will allow finding if the collection period of the plant influences the demonstrated antiviral activity of the bark decoction of *E. fusca* against HSV type 1 and 2.

Decoction and maceration extracts from bark, collected in April/2001 were evaluated in vitro against HSV-1. Negative results were obtained for both extracts.

Many alkaloids have been studied for their antiviral activity. Schumannificin and O-demetylbuchenavianin are alkaloids isolated from *Schumanmiphyton magnificum* and *Buchenavia capitata*, respectively. These compounds are active against HSV and HIV, respectively [22]. Other alkaloids have been assayed against different virus such as Cytomegalovirus (CMV) with optimal results [23]. For that reason, the low levels of alkaloids detected in aqueous extract (see Table 2) could be one of the principal reasons of inactivity of these extracts.

The collection period of the *E. fusca* bark influence the antiviral activity against HSV-1. The low cytotoxicity of bark decoction (CC_{50}>8 µg/mL) and the SI (>33 and >73, Table 3) demonstrated that the *E. fusca* bark is a potential antiviral candidate. SI higher than 10 indicated the assayed sample is sufficiently selective to inactivate the virus without greatly affecting the host cell [24]. The isolation and purification of secondary metabolites starting from bark of *E. fusca* collected in September/2000, guided by antiviral bioassays, will be the continuity of this preliminary study.

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