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Biological screening of plants of the Venezuelan Amazons

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Abstract

A total of 21 extracts derived from 17 different plant species collected in Venezuelan Amazons have been tested for the following biological activities: cardiovascular activity, brine shrimp lethality, and inhibitory effects on the hydrolysis of glucose-6-phosphate in intact and disrupted microsomes. Eight extracts diminished rat blood pressure with or without changes in heart rate. The fruit extract of *Swartzia leptopetala* and the leaf and twig extract of *Connarus lambertii* resulted in death of experimental animals. The majority of extracts (17 extracts) showed significant toxicity against *Artemia salina*. Concerning the hydrolysis of glucose-6-phosphate, better inhibitory effects were observed in intact microsomes than in disrupted ones for all the extracts, suggesting that these extracts intervene with variable potency in glucose-6-phosphate transport through the microsomal membrane. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Amazonian plants; Brine shrimp lethality test; Cardiovascular activity; Glucose-6-phosphatase; Biological screening

1. Introduction

The Neotropics is the richest region of plants in the world, counting about 90 000 higher plant species. However little is known, in comparison with other regions, from the point of view of the flora, and it is considered that 10 000 neotropical species have not yet been registered (Gentry, 1986). The majority of them are encountered in the neotropical rain forests, and in Venezuela, these rain forests cover 314 400 km^2 in the Bolivar and Amazonas States, the latter located in the southernmost part of the country, sharing the border with the Republics of Brazil and Colombia. This territory is 93% covered by thick forests, being very attractive due to its endemic plant diversity (Steyermark, 1979). As part of the Amazone project an agreement has been signed between our University and the Amazone local Government, and since 1978 one of us (A. Castillo) started to compile the taxa of the rain forest around the Cataniapo river, 8 km southeast from Puerto Ayachucho City, capital of the Amazonas State (Fig. 1). The mentioned area was selected for field work, because of its easy access to the place, being near to the capital city and with relatively few botanical collections previously done. Besides the mentioned facts, by that time there was a plan to construct a dam in the cited region and an inventory of the plant species was required before definite destruction of the area.

For that purpose, Castillo's group collected 906 numbers of plant specimen, which belonged to 95 families, 329 genera and 438 species, during various sample collection trips in the area (Castillo, 1992), and in some of these trips plants were collected for the biological screening plan. In total, 100 species of 55 families were collected, and subjected to sample preparation for posterior biological tests. In this paper we present primary results obtained from 17 different species belonging to

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13 families, Acanthaceae to Cyperaceae in alphabetical order, engaged for three following bioassays: (1) cardiovascular assay in order to find out antihypertensive substances; (2) brine shrimp lethality test which has been used successfully to detect biologically active substances (McLaughlin et al., 1991); (3) microsomal glucose-6-phosphatase assay, which could be a useful method to find out substances to reduce glucose concentration in blood, being one possible target for diabetes chemotherapy (Gonzalez-Mujica et al., 1998).

2. Materials and methods

2.1. Plant collection

Plants used were collected in the area above indicated, cartographically situated between 5° 36′–5° 39′ N and 67° 6′–67° 29′W, with an altitude of 80–250 m above sea-level (Fig. 1). Voucher specimens are deposited in the National Herbarium of Venezuela (VEN) and the Ambiental Ministry Herbarium at Puerto Ayachucho City (TFAV).

2.2. Extract preparation

The collected fresh plants were kept individually in cloth bags until the end of every day field work, then chopped into pieces, put full in a glass bottle of 500 ml capacity, filled with 95% ethanol, and stored for two months at room temperature to accomplish exhaustive extraction. The ethanolic extract was decanted and concentrated in vacuo to dryness. The dry ethanolic extract was treated with distilled water and water soluble part was freeze-dried. The freeze-dried preparation thus obtained, named extract, was kept in a glass vial at

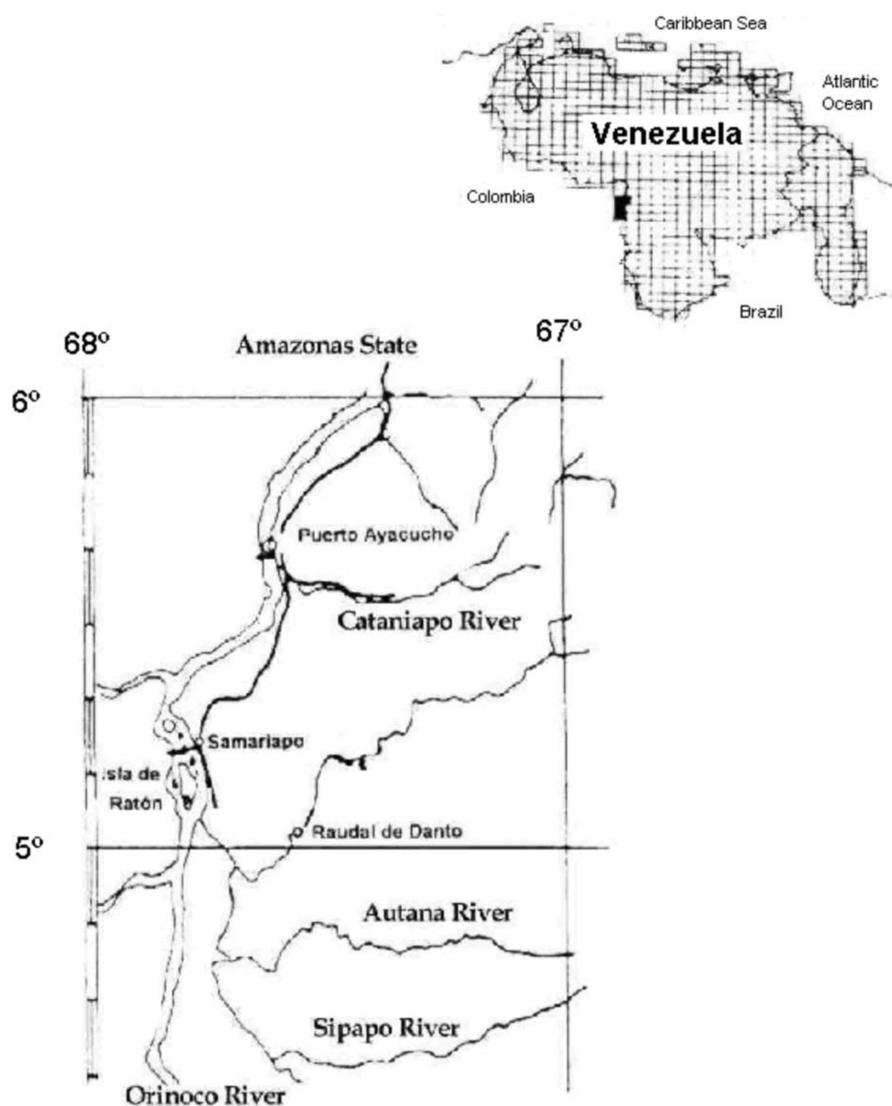

Fig. 1. Map of the place where the plant samples were collected.

−20 °C until biological assays were performed. This extract preparation procedure was not controlled quantitatively.

2.3. Biological assays

2.3.1. Cardiovascular assay

Male and female Sprague Dawley rats (250–300 g, $n=6$) were anaesthetized with sodium pentobarbital (40 mg/kg). The trachea was exposed and cannulated with a polyethylene catheter to avoid ventilation disturbances, a femoral vein catheter was introduced for extract administration. Intrarterial blood pressure was recorded from the femoral artery through a catheter connected to a strain-gauge pressure traducer connected to a polygragh (Letica, Spain). Heart rate (HR) was continuously recorded with a tachograph (beats/min) driven by the arterial pulse waves. Mean arterial blood pressure (MABP) expressed in mmHg was calculated by the sum of a 1/3 pulse pressure to the diastolic blood pressure. MABP and HR values were registered after administrating slowly 0.3 ml of 0.9% NaCl solution, and considered as the basal values. The changes on MABP and HR caused by the extract administration were evaluated once these parameters remained constant for 30 min. Sample preparation for intravenous administration was made by dissolving 10 mg of each extract in 0.05 ml of dimethylsulfoxide and 0.3 ml of 0.9% NaCl solution. The sample (30 mg/kg) was injected intravenously in a lapse of 30 s in the rat and the effects were recorded until full recovery of basal hemodynamic values. Differences between doses versus basal values were evaluated by Student's t-test for paired samples at a level of significance $P<0.05$.

2.3.2. Brine shrimp lethality test

Brine shrimp lethality test was performed by the method of McLaughlin et al. (1995). Positive control test was done using caffeine whose LC_{50} is ~ 300 ppm (Meyer et al., 1982). For the blank test, no lethality of brine shrimp was observed.

2.3.3. Glucose-6-phosphatase assay

Glucose-6-phosphatase (G-6-Pase) tests were carried out using intact and disrupted (histone treated) microsomes by the method of Burchell et al. (1988). In this work inorganic phosphate ion (Pi) resulting from the hydrolysis of glucose-6-phosphate (G-6-P) was converted to the blue complex with ammonium heptamolybdate in acidic medium and measured at 820 nm using a Novaspec II (Pharmacia) photometer. The results of bioactivity are mean values from triplicate experiments and expressed by % inhibition of the hydrolysis of G-6-P, calculated by the following equation.

$$\% \text{ Inhibition} = (EA_{control} - EA_{extract}) \times 100/EA_{control},$$

where $EA_{control}$ and $EA_{extract}$ represent the enzymatic activity in the absence or in the presence of plant extract, respectively. The EA is expressed as μmol Pi/h/mg microsomal protein. The microsomal protein concentration was estimated by a modification of the Lowry method (Markwell et al., 1978).

2.4. Phytochemical screening

To have an idea of possible classes of compounds present in the extracts, preliminary phytochemical analysis was carried out through thin layer chromatography on silica gel plates developed with a mixture of chloroform–methanol (8:2). Spots were revealed by the following spray-reagents: Dragendorff reagent for alkaloids, and 2% methanol solution of ceric sulfate-saturated concentrated sulphuric acid; the plates were heated with a hair-dryer, the presence of triterpenoids suggested by violet spots and flavonoids by yellow or orange spots (Bilia et al., 1996). The observation of orange spots and red spots by spraying the chromatograms with Natural product reagent (1% methanolic 2-aminoethyl diphenylborate) (Wagner et al., 1984) is indicative of the presence of 3′,4′-dihydroxyflavonols and 3′,4′,5′-trihydroxyflavonols, respectively (Bilia et al., 1996).

3. Results

A total of 21 extracts derived from 17 different plant species collected in Venezuelan Amazons (Table 1) were subjected to biological screening: cardiovascular activity (Table 2), brine shrimp lethality, and effects on the enzymatic activity of glucose-6-phosphatase (Table 3).

3.1. Cardiovascular activity

Fruit extract of *Swartzia leptopetala* killed experimental animals during cardiovascular bioassays, while the extract prepared from leaves and twigs of the same plant did not give any activity. Seven extracts obtained from leaves and twigs of seven different plants diminished rat arterial blood pressure by 13–49%, with or without altering the heart rate. The extract of *Connarus lambertii* killed experimental animals (Table 2). No cardiovascular activity was observed for the other 13 extracts.

3.2. Brine shrimp lethality

All the extracts except *Malouetia tamaquarina* (L-T), *Campsiandra comosa* (L-T), *S. leptopetala* (L-T), and *Rhynchospora pubera* (L-T), showed significant lethality against brine shrimp, which has been successfully used as a simple biological test, to guide fractionation pro-

Table 1
Plant species screened and results of phytochemical analysis

Species (Family)	Voucher number	Plant part[a]	Class of compounds[b]
Justicia. cataractae Leonard (Acanthaceae)	AC 1368	L-T	1, 2, 2A
Tapirira guianensis Aublet (Anacardiaceae)	AC 1641	L-T	1, 2
Guatteria maypurensis H.B.K. (Annonaceae)	AC 1579	L–T	1, 2, 3
Forsteronia laurifolia (Benth.) A. DC. (Apocynaceae)	AC 1586	L–T	1, 2, 2A
Himatanthus attenuatus (Benth.) Woodson (Apocynaceae)	AC 1560	L–T	1, 2
Malowetia tamaquarina (Aublet) A. DC. (Apocynaceae)	AC 1531	L-T	1, 2, 2A
		F	2
Xanthosoma pilosum C. Koch et Augustin (Araceae)	AC 1381	L–T	1, 2
Cordia polystachya H. B. K. (Boraginaceae)	AC 1223	L–T	2
Protium unifoliolatum Engl. (Burseraceae)	AC 1370	L–T	1, 2, 2A
		F	1, 2
Campsiandra comosa var. *laurifolia* (Benth.) Cowan (Caesalpiniaceae)	AC 1301	L–T	1, 2, 2A
Swartzia leptopetala Bentham (Caesalpiniaceae)	AC 1522	L–T	1, 2, 2A
		F	1, 2
Caryocar microcarpum (DC.) Sagot (Caryocaraceae)	AC 1635	L–T	1, 2, 2A
Buchenavia reticulata Eichler (Combretaceae)	AC 1272	L–T	1, 2, 2A
Mikania parviflora (Aublet) Karsten (Compositae)	AC 1575	L-T	1, 2, 2A
Connarus lambertii (DC.) Sagot (Connaraceae)	AC 1616	L–T	1, 2, 2B
Rhynchospora pubera (Vahl) Boeck. (Cyperaceae)	AC 1216	L–T	1, 2, 2A
Scleria cyperina Kunth (Cyperaceae)	AC 1342	L–T	1, 2, 2A
		F	1, 2, 2B

[a] L-T, Leaves and Twigs; F, Fruits.
[b] 1, Triterpenoid; 2, Flavonoid; 2A, 3′,4′-Dihydroxyflavonol; 2B, 3′,4′,5′- Trihydroxyflavonol; 3, Alkaloid.

cess of plant extracts in order to detect antitumor and pesticidal compounds (McLaughlin et al., 1991): This test is positively correlated to 9 kB cell toxicity (McLaughlin et al., 1995). Twelve out of 21 extracts screened showed LC_{50} values smaller than 100 ppm (μg/ml), being LC_{50} of the extract of *Caryocar microcarpum* (L-T) lower than 10 ppm (Table 3). LC_{50} values < 1000 ppm are considered significant for crude extracts (Meyer et al., 1982).

3.3. Glucose-6-phosphatase enzymatic activity

Without exception, all the extracts inhibited, to a greater or lesser degree, microsomal G-6-Pase enzymatic activity. In all cases, greater inhibition values were observed on intact microsomes than on disrupted ones. This fact means that the extract causes an inhibitory action not only on G-6-Pase catalytic subunit but also intervenes in the transport of G-6-P into the microsomes or in the flow-out of phosphate ion and/or glucose, the hydrolysis products of G-6-P in microsomes. Marked effects were observed for the extracts of *Tapirira guianensis* (L-T), *Forsteronia laurifolia* (L-T), and *C. lambertii* (L-T) in both intact and disrupted microsomes (Table 3).

3.4. Phytochemical analysis

Phytochemical analysis to have an idea of possible classes of compounds present in the extract was carried out by observing coloured spots revealed on thin layer silica gel plate with the use of special spray-reagents mentioned in the experimental section (Section 2.4). Almost all extracts showed violet and yellow-orange spots on the plate with ceric sulfate–sulphuric acid reagent indicating possible presence of triterpenes and flavonoids (Bilia et al., 1996). The majority of the spots corresponding to flavonoids yielded orange color by spraying Natural product reagent, indicative of 3′,4′-dihydroxyflavonols, and for the extracts of *C. lambertii* (L–T) and *Scleria cyperina* (F) red spots were observed with the same reagent, typical of 3′,4′,5′-trihydroxyflavonols (Bilia et al., 1996). The only one extract of *Guatteria maypurensis* (= *G. calva*) leaves and twigs, yielded characteristic orange spots by Dragendorff reagent, and in fact we isolated from the leaves of this plant oxoaporphines (Rodríguez et al., 1999), besides protoberberine and tetrahydroprotoberberine alkaloids (unpublished results). The results of phytochemical analysis are presented in Table 1.

4. Discussion and conclusions

In eight extracts positive for hypotensive effects (Table 2) no alkaloid was detected by Dragendorff reagent, while triterpenoids and flavonoids were common to all these extracts (Table 1). It has been reported that 4′-flavonols show correlation with cardiovascular activities (Funayama and Hikino, 1981; Duarte et al., 1993; Itoigawa et al., 1999).

The majority of the extracts submitted to brine shrimp lethality test (17 of a total of 21 extracts) showed significantly meaningful LC_{50} values (Table 3), which positively correlate to 9 kB cell toxicity (McLaughlin et al., 1995). McLaughlin et al. (1991) demonstrated the general utility of this assay to guide phytochemical screening and fractionation procedure to isolate a variety of bioactive compounds. David et al. (1998) reported the isolation of two cytotoxic compounds, 2-[10(Z)-heptadecenyl]-1,4-hydroquinone and (4R,6R)-dihydroxy-4-[10(Z)-heptadecenyl]-2-cyclohexanone, from a methanolic extract of seeds of *T. guianensis*. In our experiments *T. guianensis* (L-T) showed a value of $LC_{50} = 213$ ppm. From the wood of *Protium unifoliolatum* (in our work $LC_{50} = 75$ ppm for leaves and twigs, $LC_{50} = 25$ ppm for fruits) arylnaphthalene lignans have been isolated (Siani et al., 1998). As well, from the whole plant of *Justicia* species diarylbutane lignans and arylnaphthalide lignans have been isolated, reporting that such compounds are cytotoxic, antiviral and antiplatelet (Joseph et al., 1988; Asano et al., 1996; Chen et al., 1996; Day et al., 1999). *G. maypurensis* H.B.K. (= *G. calva* R.E.Fr.) is the only plant whose extract shows positive result to Dragendorff reagent. From the leaves of this plant we isolated three known cytotoxic oxoaporphine alkaloids, namely oxostephanine, oxoxylopine, and oxoputerine (Rodríguez et al., 1999). To our knowledge, there are no reports about phytochemical studies on the rest of the plant species listed in Table 1, relating them to the bioactivities assayed in the present work. However, the isolation of antibacterial hydroperoxysterols from the aerial parts of *Xanthosoma robustum* (Kato et al., 1996), as well as antifungal and larvicidal naphthoquinones form the roots of *Cordia curassavica* (Ioset et al., 2000), antibacterial isoflavones from the heartwood of *Swartzia polyphylla* (Osawa et al., 1992), anti-HIV and cytotoxic flavonoid-piperidine alkaloids from *Buchenavia capitata* leaves (Beutler et al., 1992), cytotoxic kaurenic acid-type diterpenes and flavone from the aerial parts of *Mikania hirsutissima* (Ohkoshi et al., 1999), and trypanocidal kaurenic acid from the aerial parts of *Mikania obtusata* (Alves et al., 1995) is known. Now, flavonoids are detected in all the extracts studied (Table 1), which are known to exhibit a variety of biological activities including inhibition of malignant cell growth and proliferation. These activities are thought to be, in part, due to inhibition of various enzymes (Agullo et al., 1997). In this context it is significant that DuBois and Sneden (1995) isolated from the heartwood of *S. polyphylla* five known flavonoids and one new prenylated isoflavone, guided by an assay for inhibition of protein kinase C.

On the other hand, triterpenoids are also detected in all the plant extracts. Triterpenes and their glycosides (saponins) have a wide distribution in the plant kingdom and display a variety of biological activities. Triterpenes of oleanane, ursane and lupane skeletons, such as oleanolic acid, ursolic acid and betulinic acid, are commonly encountered in plants, and they are known to have antitumor activity (Hsu et al., 1997; Fulda et al., 1999).

G-6-Pase is the enzyme that catalyses the last step of neoglucogenesis and glycogenolysis, and its structure has been elucidated as five polypeptide subunits (Burchell and Waddell, 1991). Its inhibition might be of some help in the control of the hyperglycaemia present in diabetes. Gonzalez-Mujica et al. (1998) reported that an aqueous extract of *Bauhinia megalandra* Griseb leaves, one of the Venezuelan folklore medicines in the treatment of diabetes, produced a 32% decrease in microsomal G-6-Pase activity. In fact, we isolated two flavones from the leaves of the mentioned plant, which suppressed the hydrolysis of G-6-P more than 98% in intact microsomes (unpublished results). In view of the results obtained with *B. megalandra* on the G-6-Pase activity, we consider 25–30% inhibition as significant for a crude extract. To our knowledge, there have not been reports concerning any of the plants cited in Table 1, that affect G-6-Pase enzymatic activity. Somewhat

Table 2
Extracts displaying positive results in cardiovascular assay

Species (plant part)	ABP[a] diminution (%)	Change of HR[b] (beats/min)	Duration (min)
Justicia cataractae (L-T)	13.2	0	15
Tapirira guianensis (L-T)	38.5	0	20
Forsteronia laurifolia (L-T)	16.2	−20	10
Himatanthus attenuatus (L-T)	35.5	0	40
Swartzia leptopetala (F)	–	–	∞[c]
Buchenavia rerticulata (L-T)	49.2	0	15
Connarus lambertii (L-T)	42.3	−10	∞[c]
Scleria cyperina (L-T)	18.5	−120	30

[a] Arterial blood pressure.
[b] Heart Rate.
[c] Not recovered.

Table 3
Results of brine shrimp lethality test and microsomal enzymatic assays

Species (plant part)	BS[a] test (LC_{50} in ppm)	G-6-P[b] hydrolysis inhibition (%)	
		Intact microsomes	Disrupted microsomes
Justicia cataractae (L-T)	100	7.6	1.8
Tapirira guianensis (L-T)	213	94.9	69.6
Guatteria maypurensis (L-T)	90	84.7	57.1
Forsteronia laurifolia (L-T)	330	93.9	72.1
Himatanthus attenuatus (L-T)	28	86.1	31.0
Malouetia tamaquarina (L-T)	>1000	22.8	2.0
(F)	42	70.5	34.5
Xanthosoma pilosum (L-T)	106	14.9	4.8
Cordia polystachya (L-T)	534	12.8	0
Protium unifoliolatum (L-T)	75	89.5	36.6
(F)	25	17.2	9.9
Campsiandra comosa var. *laurifolia* (L-T)	>1000	63.4	23.5
Swartzia leptopetala (L-T)	>1000	55.2	21.7
(F)	22	45.8	39.6
Caryocar microcarpum (L-T)	<10	88.5	34.1
Buchenavia reticulata (L-T)	50	93.8	66.1
Mikania parviflora (L-T)	80	42.5	14.7
Connarus lambertii (L-T)	155	97.5	86.2
Rhynchospora pubera (L-T)	>1000	3.6	0
Scleria cyperina (L-T)	45	92.9	45.3
(F)	50	50.0	20.0
Caffeine	360		
Phloridzin		58.0	10.0

[a] Brine shrimp.
[b] Glucose-6-Phosphate.

surprisingly, nine of a total of 21 extracts exhibited in intact microsomes, more than 80% inhibition of hydrolysis of G-6-P. In the case of *Himatanthus attenuatus* (L-T), *Protium unifoliolatum* (L-T), *Caryocar microcarpum* (L-T) and *Selerina cyperina* (L-T), the inhibition percentage of enzymatic activity in disrupted microsomes was about half as much as the inhibition percentage in intact microsomes. This means that about one half of the inhibition in intact microsomes should be correlated to the inhibition of G-6-P transport inside the microsomes (T1 Transporter) and/or flow-out of phosphate ion and/or glucose outside of the microsomes (T2 and/or T3 transporter). On the other hand, the extracts derived from *T. guianensis* (L-T), *Forsteronia laurifolia* (L-T), *Buchenavia reticulata* (L-T) and *C. lambertii* (L-T), showed higher inhibition of enzymatic activity on disrupted microsomes than the previously mentioned group of plants. Therefore, the extracts not only inhibit the G-6-Pase system transporters (T1, T2, and T3) but also enter the microsomes and inhibit the action of the G-6-Pase catalytic subunit preventing the hydrolysis of the G-6-P, being the first mentioned group of plants more effective as inhibitors of the transporters and the second one of the catalytic subunit.

At the end of our discussion it must be kept in mind that the compounds present in the extracts are water-soluble substances, therefore triterpenoids and flavonoids commonly detected in the extracts might be present in glycosidic forms. On the other hand, it should be considered that other classes of compounds, i.e. tannins, particularly in relation to G-6-Pase enzymatic activity, carbohydrates, amino acids and their derivatives, and any other polar substance can be responsible for biological activities assayed in this work.

In conclusion, Amazonian biodiversity is huge, but in danger by continuous and uncontrolled deforestation and other damages caused day by day. Even though a number of groups in the world are working on Amazonian plants, there remains much to be done. During the course of our phytochemical studies on plants of the Venezuelan Amazons, we collected 100 species belonging to 55 families to prepare sample extracts for biological screening. In this paper the results obtained from 17 plant species of 13 families are presented, and we are continuing to realize biological screening for the rest of plants. We hope our work can serve for any future ethnobotanical and phytochemical research.

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