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Pharmacological activity and chemical composition of callus culture extracts from selected species of *Phyllanthus*

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This study was conducted in order to determine the chemical composition and the possible antinociceptive effects in mice of some species of *Phyllanthus in vitro*. The methanolic extracts obtained from callus cultures of *P. fraternus*, *P. stipulatus* and *P. caroliniensis* caused significant inhibition in to the late phase of the formalin test, whereas the extract from *P. urinaria* inhibited both neurogenic and inflammatory phases of the test. Conventional chromatographic methods (TLC, GC) permitted the detection of some steroids or triterpenes, including β-sitosterol, glochidonol and glochidone, which seem be responsible for the antinociceptive effects of the callus extracts studied.

1. Introduction

The plants of the genus *Phyllanthus* are popularly known in Brazil as "Quebra pedra", "Erva pombinha" or "Arrebenta pedra". Infusion of leaves, stems and roots of most of these species have been used in folk medicine for the treatment of several diseases, including disorders of the kidney and urinary bladder, intestinal infections, diabetes and hepatitis B virus infections [1–3].

A great variety of these plants have been investigated phytochemically and pharmacologically and many molecules have been isolated and identified. Several compounds, including alkaloids, flavonoids, lignans, phenols, steroids and terpenes exhibited potential therapeutic benefit in the management of hepatitis B, nephrolithiasis and in painful disorders [4].

In continuation of our investigations of the genus *Phyllanthus*, we have selected now some species cultivated *in vitro* [5] i. e*., P. fraternus, P. caroliniensis, P. urinaria* and *P. stipulatus* and evaluated their chemical composition as well as their antinociceptive effects in mice.

2. Investigations, results and discussion

Callus cultures were initiated from single node cuttings (± 10 mm lengths) excised from 5–6 weeks old *in vitro* grown plants and transferred to a MS basal medium supplemented with 2,4-D (4.4 μM) for *P. caroliniensis, P. stipulatus* and *P. fraternus* and IBA (40 μM) and BAP (9 μM) for *P. urinaria* (Table 1). The lyophilised callus of different *Phyllanthus* species were macerated with methanol for five days to obtain the respective methanolic extracts. The extract yields, (%, related to dry weight) were 14.8, 12.7, 15 and 42% for *P. caroliniensis, P. stipulatus, P. urinaria* and *P. fraternus,* respectively. All the extracts were analysed by TLC using several solvent system (eluants) and specific reagents [6, 7]. The results indicated that the extracts do not contain alkaloids or phenolic compounds (tannins, flavonoids, etc.) in detectable concentrations (TLC). However, the strong positive reaction with anisaldehyde-sulfuric acid reagent suggested the presence of several steroids or terpenoids. One of the main compounds was determined (by co-TLC and co-GC) to be β-sitosterol, a well documented phytosterol, which was previously detected in the methanolic extracts of *Phyllanthus* [8]. It exerted significant antinociceptive effects when evaluated in different models of pain in mice, being equipotent to aspirin or paracetamol in writhing and formalin tests in mice [9]. Other triterpenes were determined in these extracts, such as glochidiol, glochidonol and glochidone, which also were found previously in the *P. sellowianus* roots [10]. The latter was recently isolated from *Ipomea pes-caprae* (L.), exhibiting considerable analgesic action in mice [11]. The presence of glochidonol in all the callus extracts was confirmed by TLC and GC, whereas glochidone was only detected in *P. caroliniensis* and *P. fraternus.* β-Sitosterol was observed in all the extracts except *P. caroliniensis.* Other triterpenes and sterols were verified in callus extracts through TLC and GC procedures, but they are not yet recognisable. The presence of these compounds in callus indicate the possibility to improve and increase the production *in vitro.* The optimized callus culture protocols now offer the possibility to use cell culture techniques for vegetative propagation and open the door for further studies on secondary metabolites. The results presented in Table 1 show that methanolic extracts of callus obtained from *P. fraternus, P. stipulatus* or *P. caroliniensis,* given intraperitoneally, produced significant inhibition in relation to the second phase of the formalin-induced licking. In contrast, the methanolic extract obtained from *P. urinaria* caused significant inhibi-

Table 1: Callus fresh weight of *Phyllanthus* species of cultivated on medium MS supplemented with auxins under dark culture

Auxin	Fresh weight (mg)			
	P. caroliniensis	*P. stipulatus*	*P. urinaria*	*P. fraternus*
2,4-D (4.4 μM)	134.45 ± 47.5	230.04 ± 53.03		91.83 ± 23.68
BAP (9 μM) + IBA (40 μM)			473.28 ± 77.08	

Data are the mean of twenty four replicates.
2,4-D: Dichlorophenoxyacetic acid; IBA: Indole-3-butyric acid; BAP: 6-bensylaminopurine

Table 2: Antinociceptive action of methanolic extracts from callus of *Phyllanthus* species, aspirin and acetaminophen in formalin-induced licking in mice (30 mg/kg, i.p.)

Treatment	Formalin- induced licking	
	Inhibition (%) First Phase (0–5 min)	Inhibition (%)[b] Second Phase (15–30 min)
Control	0	0
P. fraternus[a]	15.0 ± 6.0	44.0 ± 7.0**
P. stipulatus[a]	25.0 ± 9.0	65.0 ± 6.0**
P. caroliniensis[a]	24.0 ± 8.0	45.0 ± 4.0**
P. urinaria [a]	46.0 ± 5.0**	57.0 ± 7.0**
Acetaminophen[b]	15.0 ± 3.0	56.0 ± 6.0**
Aspirin[b]	16.0 ± 2.0	45.0 ± 5.0**

Each value represents the mean of the four to six animals. [a] Methanolic extract. [b] Data from Vaz et al., 1996. The asterisks (** $p < 0.01$) denote the significance level when compared with control group.

tion of both phases of the formalin test (Table 2). Relevant are also the results showing that the methanolic extracts from *Phyllanthus* species were almost equally efficacious when compared with non-steroidal antiinflammatory drugs (aspirin and acetaminophen) against the second phase of formalin-induced licking.

In summary, our results indicate that the experimental conditions for callus establishment produce mainly sterols and triterpenes, which seem to be responsible for the antinociceptive effect of the methanolic extracts.

3. Experimental

3.1. Plant material

Seeds of *P. fraternus, P. stipulatus* and *P. urinaria* were collected in the gardens of the Federal University of Santa Catarina (UFSC, Florianópolis, SC), and seeds of *P. caroliniensis* were collected in Urussanga, Brazil. They were classified by Dra. Leila da Graça Amaral and Ms. Miriam Ulyssea (Department of Botany, UFSC, Brazil). A voucher specimen of each species was deposited in the herbarium FLOR (Department of Botany, UFSC).

3.2. Establishment of "in vitro" cultures

Seeds of *P. fraternus* and *P. stipulatus* were surface sterilised with immersion in 2% sodium hypochorite for 20 min., and then rinsed 4−5 times with sterile distilled water. Single node cuttings were obtained from aseptically cultured *P. caroliniensis* and *P. urinaria*. They were germinated and cultivated in hormone free medium MS (Murashige & Skoog, 1962), 2% (w/v) sucrose and 0.2% Phytagel. The medium was adjusted to pH 5.8 prior to autoclaving (18 min at 120 °C).

3.3. Callus culture

The explants were cultivated in 60 ml tubes containing 8 ml of culture medium, were kept in a culture room at 25 ± 2 °C in total darkness. The explants of *P. fraternus* and *P. stipulatus* were taken from seedling 30 days after germination, and explants of *P. caroliniensis* and *P. urinaria* were taken from plants axenics after 40 days cultured (shoot tips with ± 10 mm lengths). To obtain callus development, 4 μM 2,4-dichlorophenoxyacetic acid (2,4-D) for *P. caroliniensis, P. fraternus and P. stipulatus* and 40 μM indole-3-butyric acid (IBA) and 9 μM 6-bensylaminopurine (BAP) for *P. urinaria* were added to medium MS. All the cultures were incubated in growth medium for approximately 50 days and then were harvested for phytochemical analysis.

3.4. Phytochemical analysis

Callus fresh tissues were lyophilised and macerated with methanol at room temperature for approximately 10 days. After solvent evaporation, the chromatographic profile of all the methanolic extracts was analysed by TLC using Merck silica precoated aluminium plates 20 μM in thickness using $CHCl_3$: MeOH 9 : 1 as eluent. Spots were visualised by general and specific reagents (UV radiation, $FeCl_3$, anisaldehyde-sulfuric and Dragendorff reagent) according to previously described methods [6, 7]. The samples were also analysed by GC. The chromatographic analysis were performed on a GC-14A Shimadzu equipped with a 30 m × 0.25 mm i.d. column coated (0.3 μm film thickness) with cross-linked polymethylsiloxane as stationary phase (column LM-1, by L&M, São Carlos, Brazil). Samples were introduced using the "plitless mode" (1 min, 1.0 μl injection volume), with flame ionisation detector (FID, temperature 320 °C) and column temperature programming from 40 °C to 310 °C (40 °C to 240 °C at 12 °C · min^{-1} and 240 °C to 310 °C at 5 °C · min^{-1} and held for 20 min). Hydrogen was used as the carrier gas. Data were processed using the Cromatografia program (Microquímica), on a 486PC. The standard compounds were previously isolated from this genus [10]. The presence of glochidone, glochidonol, glochidiol and β-sitosterol, was confirmed by co-TLC and co-GC with authentic samples.

3.5. Pharmacological analysis

The antinociceptive action of the methanolic extracts was investigated in the formalin-induced test licking in mice according to the procedure described previously [8, 12]. 20 μl of 2.5% formalin solution (0.92% formaldehyde) were injected intraplantarly in the right hindpaw. Male Swiss mice (25–30 g) received the methanolic extracts from *Phyllanthus* species (30 mg/kg) or vehicle (0.9% NaCl, 10 ml/kg) given intraperitoneally 30 min before formalin injection.

3.6. Statistical analysis

The pharmacological results are presented as mean ± s.e.m, and statistical significance between the groups was analysed by means of *t* test or analysis of variance followed by Dunnett's multiple comparison test, when appropriate. *P* values less a 0.05 were considered as indicative of significance.

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