Laboratório de Fisiologia Vegetal[1], Instituto de Biofisica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, RJ, and Núcleo de Investigações Químico-Farmacêuticas (NIQFAR)[2], Curso de Farmácia-CCS, Universidade do Vale do Itajaí (UNIVALI), Itajaí, SC, Brazil

Pharmacological and phytochemical studies of callus culture extracts from *Alternanthera brasiliana*

A. F. Macedo[1], N. C. Barbosa[1], M. A. Esquibel[1], M. M. Souza[2] and V. Cechinel-Filho[2]

This work describes the establishment of callus cultures of *Alternanthera brasiliana*, a Brazilian medicinal plant used to treat several ailments. In addition the two extracts, exhibiting best yields, were chemically analysed and evaluated as antinociceptive agents in two classical models of pain in mice: the writhing test and the formalin test. The results show that the highest biomass accumulation was observed in callus grown in media with higher concentrations of growth regulator of 2,4-D. Both extracts studied exhibited antinociceptive effects in mice, being more effective than the plant extracts. The pharmacological action seems to be related to the presence of steroids and/or terpenes.

1. Introduction

Alternanthera brasiliana L. Kuntze (Amaranthaceae), known as "terramicina" or "penicilina" is a medicinal plant which is widely used in Brazilian folk medicine to treat some diseases, including inflammation, pain and infectious processes [1]. Although phytochemical investigations concerning this species are rare, some authors have demonstrated that the genus *Alternanthera* contains mainly steroids [2, 3] and flavonoids [4–6]. Previous studies carried out in our laboratories have shown that the hydroalcoholic extract obtained from the aerial parts of *A. brasiliana* exhibits potent antinociceptive effects when evaluated in different models of nociception in mice [3].
In this paper, we report the establishment of callus cultures of *A. brasiliana* and analysed the possible antinociceptive effects of two selected methanolic extracts against some classical models of nociception in mice. In addition, we evaluated these extracts phytochemically.

2. Investigations, results and discussion

Callus cultures were initiated from seedlings grown from seeds of *A. brasiliana*, which were placed on Murashige and Skoog's medium [7] with the addition of different growth regulators. Primary callus cultures were employed and the results indicate that the highest biomass accumulation was observed in callus grown in media with higher 2,4-D concentrations than BAP concentrations or in a medium containing 2,4-D as the only growth regulator (media D and A) (Table 1). Higher BAP concentrations (medium C) or equal 2,4-D and BAP concentrations (media E and F) decreased the accumulation of dry and fresh weight (Table 1). BAP inhibits the auxin induced accumulation of biomass on callus of *A. brasiliana*.
Results obtained with *A. brasiliana* on medium D (Table 1) and in a number of investigations [8] showed that auxin and cytokinin combinations were either necessary or beneficial.
The lyophilized callus tissues of *A. brasiliana* were macerated with methanol for seven days to obtain the respective methanolic extracts. The extract yields (%, related to dry weight) were 38.4, 43.6, 21.2, 28.5, 14.7 and 27.4 for calli from medium A, B, C, D, E and F, respectively. All extracts were analysed by TLC using several solvent systems (eluants) and specific reagents [9, 10]. 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 and Lieberman-Burchard reagents suggested the presence of several steroids or terpenoids. One of the main compounds was determined (by spectral data, co-TLC and co-GC) to be β-sitosterol, a well documented phytosterol, which was previously detected in the hydroalcoholic extract of *A. brasiliana* [3]. It exerts antinociceptive effects when evaluated in different models of pain in mice [11].
Since media A and B resulted in the best quantitative extract yields, they were selected for pharmacological analysis. Tables 2 and 3 show the antinociceptive activity of these extracts in the writhing and formalin tests at 10 mg/kg, given intraperitoneally. For purposes of comparison, we have included the data of the hydroalcoholic extract (HE) obtained from the aerial parts of *A. brasiliana* previously reported [3] and some well-known non-steroidal antiinflammatory and analgesic drugs, which were evaluated by the same experimental procedures. As can be seen, both extracts A and B significantly inhibited the abdominal constrictions, causing 66.6 and 50.4% of inhibition, respectively, being equipotent to HE and more active than standard drugs, which inhibited the abdominal constrictions by 35% (aspirin) and 38% (paracetamol).
When evaluated against formalin-induced pain in mice at 10 mg/kg, i.p., A and B showed marked antinociceptive effects in relation to the second phase, causing inhibition

Table 1: Effect of different media on biomass accumulation of *A. brasiliana* callus after 2–3 months

Media	Fresh weight (g)	dry weight (g)
A (MS + 4 μM 2,4-D)	0.66 ± 0.07 e	0.06 ± 0.02 ac
B (MS + 4 μM BAP)	0.07 ± 0.01 bc	0.03 ± 0.02 bc
C (MS + 1 μM 2,4-D + 4 μM BAP)	0.32 ± 0.03 a	0.04 ± 0.03 ac
D (MS + 4 μM 2,4-D + 1 μM BAP)	0.89 ± 0.09 d	0.08 ± 0.04 a
E (MS + 4 μM 2,4-D + 4 μM BAP)	0.32 ± 0.03 a	0.05 ± 0.03 ac
F (MS + 1 μM 2,4-D + 4 μM BAP)	0.31 ± 0.1ac	0.04 ± 0.03 ac

* Results are for 10 replicates, repeated two times with mean ± S.E. = standard error, values with different letters are significantly different ($p < 0.05$) with Tukey test. ANOVA was performed on the results of each experiment, and after confirmation of the significance of the F-value, the data means were compared using the Tukey test.

Table 2: Antinociceptive effect of methanolic extracts obtained from cultured callus (A and B), HE of *A. brasiliana* and some reference drugs on acetic acid-induced abdominal constrictions in mice (10 mg/kg, i.p.)

Treatment	Inhibition (%)
A	66.6 ± 4.4
B	50.4 ± 2.6
HE	68.8 ± 4.8
Aspirin	35.0 ± 2.0
Paracetamol	38.0 ± 1.0

Each group represents the mean ± s.e.m. of six experiments
Solvent used to dissolve the extracts did not caused any inhibition

of 94% and 75%, respectively, in relation to the control group, and were considerably more efficient than HE and indomethacin, which produced inhibition of 26% and 33%, respectively. The weak activity in relation to the first phase (neurogenic pain) is an evidence that the mechanism of action of A and B does not involve opioid receptors [12]. However, further experimental investigations are required to elucidate the mechanism concerning their antinociceptive effects.

β-Sitosterol, the main phytosterol present either in HE or A and B, also exhibits antinociceptive effects against the second phase of formalin test [11], but it is less active than the extracts studied. Thus, the phytochemical and pharmacological results reported here led us conclude that other compounds are also acting as antinociceptive agents in these extracts.

In conclusion, we report for the first time a procedure for the succesful production of *A. brasiliana* calli derived from nodal explants. The results showed the potential of these tissue cultures for β-sitosterol biosynthesis, and higher analgesic responses than "*in vivo*" plant extracts. Studies are in progress in order to determine the active principles present in callus cultures of *A. brasiliana*.

3. Experimental

3.1. Plant material

A. brasiliana was collected from a plant population growing in the gardens of Universidade Federal do Rio de Janeiro-CCS, in March 1994 and identified by the Botanic Garden of Rio de Janeiro where a voucher is registered under number RB 310.939 – A. F. Macedo 01. The plants were maintained in a greenhouse in pots (25 diam.) filled with a mixture of soil and organic matter (1:1).

3.2. Establishment of "in vitro" cultures

Seeds obtained from greenhouse-grown plants were washed with a commercial detergent for 30 min. After three washes in distilled water the seeds were surface sterilized by immersion in a 70% (v/v) solution of ethanol for 2 min, under shaking, followed by rinsing in sterile distilled water. Then the seeds were desinfected with sodium hypochlorite (50% v/v) for 20 min, followed by 5 rinses with sterile distilled water and aseptically inoculated and germinated in culture tubes containing Murashige and Skoog's [24] basal salt medium supplemented with 30 g/l sucrose, 1.48 μM thiamine, 2.43 μM pyridoxine, 4.06 μM nicotinic acid and 0.55 mM myo-inositol (MS). The pH of the medium was adjusted to 5.7 ± 0.1 before the addition of 8 g/l of agar and then autoclaved for 15 min at 120 °C and 1.1 Kgf · cm^{-2}.

3.3 Culture tests

After 50 days "in vitro" the seedlings were subcultured in the same kind of medium of germination. Nodal segments (1 cm long), with two axillary buds without petioles and leaves cut from the subcultured plantlets were placed vertically in flasks (10 cm length × 6.5 cm internal diameter; 4 explants per vessel) containing 30 ml medium. Apical and base nodal segments were discarded.

In order to obtain callus development some growth regulator combinations were added to MS medium, as follows: **A**: 4 μM 2,4-dichlorophenoxyacetic acid (2,4-D); **B**: 4 μM 6-benzylaminopurine (BAP); **C**: 1 μM 2,4-D + 4 μM BAP; **D**: 4 μM 2,4-D + 1 μM BAP; **E**: 4 μM 2,4-D + 4 μM BAP; **F**: 1 μM 2,4-D + 1 μM BAP.

All cultures were incubated in a growth chamber (25 °C, 16 h photoperiod) under illumination using day-light type fluorescent lamps (1.6 W/m^2) for approximately 2–3 months. To evaluate the dry weight, the calli were dehydrated in an oven at 40 °C for 72 h, until constant weight was obtained.

Table 3: Antinociceptive effect of methanolic extracts obtained from culture callus (A and B), HE of *A. brasiliana* and a reference drug in formalin test in mice (10 mg/kg, i.p.)

Treatment	First phase[1] Inhibition (%)	Second phase[2] Inhibition (%)
A	30.6 ± 7.2	94.0 ± 2.4
B	24.0 ± 8.0	75.0 ± 3.8
HE	20.1 ± 6.4	26.2 ± 4.3
Indomethacin	6.6 ± 1.0	33.0 ± 5.0

Each group represents the mean ± s.e.m. of six experiments
[1] 0–5 min licking (s)
[2] 15–30 min licking (s). Solvent used to dissolve the extracts did not caused any inhibition

3.4. Phytochemical analysis

The chromatographic profile of all methanolic extracts was analysed by TLC using Merck silica precoated aluminium plates 200 μm in thickness with different solvent systems ($CHCl_3$/MeOH and Hexane/AcOEt). Spots were visualized by general and specific reagents (UV radiation, sulfuric acid/methanol, $FeCl_3$, anisaldehyde-sulfuric and Dragendorff reagent) according to previously described methods [9, 10]. The presence of β-sitosterol, which was purchased commercially, was confirmed by GC.

3.5. Pharmacological analysis

The antinociceptive activity was evaluated in the writhing test [3, 11] and the formalin-induced-pain test [3, 11, 12] in mice according procedures previously described. Male Swiss mice (25–30 g) were used. The extracts were dissolved in 0.9% NaCl at 10 mg/kg (10 mg dry matter in the extract) and standard drugs were dissolved in 0.5% Tween® 80.

3.6. Statistical analysis

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

Acknowledgements: The authors wish to thank the academic and technical staff of the Laboratório de Tecido Conjuntivo-Hospital Universitário/UFRJ, mainly Ana Paula P. Alves and Claudio C. Werneck for lending the equipments. This work was supported by grants from CNPq/RHAE, CAPES and ProPPEX/UNIVALI.

References

1 Araújo, D. S.; Viana, G. S. B.; Matos, F. J. A.; Néri, E. D. R.: XII Simpósio de Plantas Medicinais do Brasil, p. 98, Fortaleza, Brazil 1994
2 Patterson, G. W.; Xu, S.; Salt, T. A.: Phytochemistry **30**, 523 (1991)
3 Souza, M. M.; Kern, P.; Floriani, A. E. O.; Cechinel Filho, V.: Phytother. Res. **12**, 279 (1998)
4 Richardson, M.: Biochem. Syst. Ecol. **6**, 283 (1978)
5 Soares, G. L. G.; Kaplan, M. A. C.: Rev. Bras. Farm. **76**, 69 (1995)
6 Brochado, C. O.; Almeida, A. P.; Costa, S. S.: XIII Reunião Anual da FESBE, p. 291, Caxambu, MG, Brazil 1998
7 Murashige, T.; Skoog, F.: Physiol. Plant **15**, 473 (1962)
8 Bhaskaran, S.; Smith, R. H.: Ann. Bot. **64**, 217 (1989)
9 Marini-Bettólo, G. B.; Nicoletti, M.; Patamia, M.; Galeffi, C.; Messana, I.: J. Chromatogr. **213**, 113 (1981)
10 Ugaz, O. L.: Investigación fitoquímica. 2. Ed., p. 10, Lima: Pontificia Universidad Catolica del Peru-Fondo Editorial 1994
11 Santos, A. R. S.; Cechinel, Filho, V.; Niero, R.; Pizzolatti, M. G.; Delle Monache, F.; Yunes, R. A.; Calixto, J. B.: Planta Med. **61**, 329 (1995)
12 Hunskaar, S.; Fasmar, O. B.; Hole, K.: J. Neurosci. Methods **14**, 69 (1985)

Received December 3, 1998
Accepted March 22, 1999

Prof. Dr. Valdir Cechinel Filho
Núcleo de Investigações Químico-Farmacêuticas (NIQFAR)
Universidade do Vale do Itajaí (UNIVALI), R. Uruguai, 458;
Cx.P. 360, 88302-202, Itajaí-SC-Brazil
cechinel@mbox1.univali.rct-sc.br