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Analgesic Effects of Callus Culture Extracts from Selected Species of *Phyllanthus* in Mice

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Abstract—The aim of this study was to evaluate the analgesic effect of the methanolic extract from callus culture of *Phyllanthus tenellus*, *P. corcovadensis* and *P. niruri* in several models of pain in mice. The extracts (medium containing 2,4-dichlorophenoxyacetic acid) of *P. corcovadensis*, *P. niruri* and *P. tenellus* (3–90 mg kg^{-1}, i.p.) caused graded inhibition of abdominal constrictions induced by acetic acid (0·6%), with ID50 (i.e. dose that reduced response of control by 50%) values of about 30, 19 and >30 mg kg^{-1}, respectively. The extract of callus of *Phyllanthus* obtained in indole-3-butyric acid and indole-3-acetic acid media (3–90 mg kg^{-1}, i.p.) caused a similar analgesic effect. In the formalin test, the extract of *P. tenellus* obtained in indole butyric acid medium (3–100 mg kg^{-1}, i.p.) inhibited only the second phase of formalin-induced pain with an ID50 value of about 100 mg kg^{-1}. Both the indole acetic acid and indole butyric acid methanolic extracts of *P. tenellus* and *P. corcovadensis* (10–100 mg kg^{-1}, i.p.) dose-dependently inhibited both phases of formalin-induced pain (ID50 values for the second phase were approx. 100 and 52 mg kg^{-1}, respectively). However, the extract of callus from *Phyllanthus* failed to affect formalin-induced paw oedema, as well as the response to radiant heat in the tail-flick test. In addition, the analgesic effect of morphine, but not the analgesic effects caused by *Phyllanthus* callus extract, was fully antagonized by naloxone. Preliminary phytochemical analysis revealed the presence of several compounds having no apparent relationship with alkaloids or flavonoids but showing the presence of phenols. These results indicate that, similar to previous reported data from the extract of *P. corcovadensis*, the methanolic extracts of callus culture of *P. niruri*, *P. corcovadensis* and *P. tenellus* exhibit potent analgesic properties against neurogenic and inflammatory pain that seem to be unrelated to the activation of opioid mechanisms.

The plants of the genus *Phyllanthus* (*Euphorbiaceae*) consist of more than 600 species that are widely distributed in tropical and subtropical countries. Infusions of leaves, stems and roots of several plants of this genus have been used for thousands of years in Brazil and many other countries as traditional remedies for the treatment of kidney and bladder calculi, diabetes, dysentry and other infections of the intestines (Morton 1981; Oliver-Bever 1983). An increasing interest in these plants has been provoked more recently by reports demonstrating that the aqueous extract of some species of *Phyllanthus* exhibit potent in-vitro and in-vivo inhibition against hepatitis B virus (Venkateswaran et al 1987; Thyagarajan et al 1988, 1990; Blumberg et al 1989; Shead et al 1992). Studies from our own laboratory have demonstrated that the extract and some alkaloids isolated from *P. sellowianus* cause a potent antispasmodic activity against several agonist-induced contractions in the guinea-pig isolated ileum and rat uterus (Calixto et al 1984). One of these compounds was identified as an alkaloid, denoted as phyllanthimide (Tempesta et al 1988). Very recently, Ogata et al (1992) demonstrated that the aqueous extract of *P. niruri* inhibits the human immunodeficiency virus type-1 reverse transcriptase (HIV-1-RT), in-vitro. The active principle responsible for its effect was isolated and identified as being the repandusinic acid A monosodium salt (Ogata et al 1992).

We have recently demonstrated for the first time that the hydroalcoholic extract obtained from the leaves, stems and roots of *P. corcovadensis*, given either orally or intraperitoneally, exhibits potent and graded antinociceptive activities in several models of nociception in mice (Gorski et al 1993). The purpose of the present study was, therefore, to evaluate the possible antinociceptive activity of the methanolic extract and some fractions obtained from callus culture of *P. corcovadensis*, *P. niruri* and *P. tenellus* in several models of nociception in mice. In addition, we have also investigated the effect of the methanolic extract of callus culture of the *Phyllanthus* species against acetylcholine-induced contractions of the guinea-pig isolated ileum. Some preliminary chemical analysis of the callus culture extracts is also reported.

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Materials and Methods

Plant material and callus initiation

Seeds of *P. tenellus*, *P. niruri* and *P. corcovadensis* were thoroughly rinsed with tap water, immersed for 15 min in 40% commercial bleach, rinsed six times in sterile distilled water and innoculated on Murashige and Skoog salts medium (MS) (Murashige & Skoog 1962) basal medium supplemented with sucrose (20 g L^{-1}) and Difco-Bacto agar (6 g L^{-1}). The cultures were exposed to fluorescent light for 16 h, and the temperature was maintained at 25 ± 2°C.

Callus initiation was achieved by culturing stem segments of 60-day-old sterile seedlings on MS medium containing sucrose (20 g L^{-1}), Difco-Bacto agar (6 g L^{-1}) and either 2,4-dichlorophenoxyacetic acid, indole-3-butyric acid or indole-

3-acetic acid at 2, 4 and 8 mg L^{-1} (Moreno & Viana, unpublished results). The cultures were incubated in the dark at 25°C for 50 days. In all cases the pH of the medium was adjusted to 5·8 before autoclaving.

Preliminary chemical analysis of callus culture

The fresh callus tissues of *P. tenellus*, *P. corcovadensis* and *P. niruri* were weighed and extracted with 95% methanol at room temperature (25°C) by maceration for 10 days. The methanolic extracts were further extracted with ethyl acetate and evaporated under reduced pressure yielding a residue of approximately 1% original weight of tissue. The methanolic extracts were partitioned with ethyl acetate and the pharmacological tests were carried out with both extracts.

The presence of flavonoid in the methanolic extracts of callus tissues was determined by the ferric chloride test and magnesium hydrochloride reduction test. The presence of alkaloid was tested by TLC with Dragendorff and Wagner reagents as described previously (Domingues 1973; Ikan 1976; Ugaz 1988). The methanolic extracts were also analysed by HPLC with detection at 254 nm by using a C-18 column eluted with methanol at a flow rate of 3 mL min^{-1}.

Pharmacological analysis

In-vivo studies. Abdominal constriction response to intraperitoneal injection of dilute acetic acid. Male Swiss mice, 25–30 g, were kept in an automatically controlled temperature (23 ± 2°C) and 12 h light-dark cycles. Food and water were freely available. The abdominal constriction induced by intraperitoneal injection of acetic acid (0·6%) was carried out according to the procedures described previously (Collier et al 1968) with minor modifications. Briefly, animals were pretreated either with the methanolic extract or with ethyl acetate extract from callus culture (3–90 mg kg^{-1}, i.p.) or with morphine (5 mg kg^{-1}, s.c.) 30 min before the acetic acid injection. In a separate group of mice, we analysed the effect of naloxone (5 mg kg^{-1}, i.p.) injected 10 min beforehand against the analgesic effect caused by both morphine and the methanolic extract of *P. corcovadensis*. Control animals received a similar volume of 0·9% NaCl (10 mL kg^{-1}, i.p.). All experiments were carried out at 20–22°C. After challenge, pairs of mice were placed in separate boxes and the number of abdominal constrictions was cumulatively counted over a period of 20 min. Antinociceptive activity was expressed as the reduction of the number of abdominal constrictions in mice pretreated with the extracts of callus culture or morphine.

Formalin-induced pain. Male Swiss mice, 25–30 g, were used. The procedure was similar to that described previously (Hunskaar et al 1985; Corrêa & Calixto 1993). Animals from the same strain were lightly anaesthetized with ether, except when used to analyse the first phase of formalin-induced pain, and 20 μL 2·5% formalin (0·92% formaldehyde) made up in phosphate-buffer was injected under the paw surface of the right hindpaw. Two mice (control and treated) were observed simultaneously from 0 to 30 min following formalin injection. The amount of time spent licking the injected paw was timed with a chronometer and was considered as indicative of pain. The initial nociceptive scores normally peaked at about 5 min after formalin injection (first phase) and 15–30 min after formalin injection (second phase), representing the neurogenic and inflammatory pain responses, respectively (Hunskaar & Hole 1987). Animals were treated intraperitoneally with the methanolic extract from callus obtained from *Phyllanthus* species (3–100 mg kg^{-1}) 30 min before formalin injection. Control animals received only the vehicle used to dilute the extracts (NaCl solution, 10 mL kg^{-1}). Following intraplantar injection of formalin, the animals were immediately placed into a glass cylinder of 20 cm in diameter, and the time spent licking the injected paw was determined. At the end of the experiments the animals were killed by cervical dislocation and the paws were cut at the knee and weighed.

Tail-flick test. Male Swiss mice, 25–30 g, were used. A radiant-heat tail-flick analgesiometer was used to measure response latencies as described by D'Amour & Smith (1941), with minor modifications. Animals responded to a focused heat-stimulus by flicking or removing their inflicted tail, exposing a photocell in the apparatus immediately below the tail. The reaction time was recorded for control mice or for animals pretreated with extract of callus from *P. tenellus* (in indole acetic acid or indole butyric acid). An automatic 8 s cut-off was used to prevent tissue damage. The animals were selected 24 h previously on the basis of their reactivity in the model. A latency period of 20 s was defined as complete analysis. The extract was administered intraperitoneally (50 mg kg^{-1}) 30 min before the trial.

In-vitro analysis. Guinea-pig ileum. Guinea-pigs of both sexes, 300–450 g, were used. The ileum was removed and preparations of 15 to 20 mm long were placed in a 5-mL organ bath containing Tyrode solution at 37°C with the following concentration (mM): NaCl 137, KCl 2·7, $CaCl_2$ 1·8, $MgCl_2$ 1·0, $NaHCO_3$ 11·9, NaH_2PO_4 0·4 and glucose 5·5. The isotonic contractions were recorded on a kymograph by using a light lever (sixfold amplification) under 1 g of tension. After the equilibration period of about 30 min, cumulative concentration-response curves were obtained for acetylcholine (0·01–100 μM) in the absence or in the presence of the extract from callus culture extracts of *P. tenellus*, *P. niruri* and *P. corcovadensis* (20–320 μg mL^{-1}) incubated with the preparations 20 min before. Control experiments were carried out in the presence of vehicle used to dilute the methanolic extract (0·9% NaCl).

Drugs

The drugs used were acetylcholine iodide (Sigma Chemical Company, St Louis, USA), formalin, acetic acid, morphine hydrochloride (Merck AG, Darmstadt, Germany) and naloxone hydrochloride (Dupont, Garden City, USA). All reagents were of a high grade of purity. All drugs and extracts were dissolved in 0·9% NaCl or in physiological buffer solution.

Statistical analysis

The results are presented as mean ± s.e.m., and statistical significance between groups was analysed by means of analysis of variance followed by Dunnett's multiple com-

Table 1. Effect of methanolic extracts of callus culture of *Phyllanthus* given intraperitoneally on acetic acid-induced abdominal constrictions in mice.

Medium	Dose (mg kg^{-1})	Number of constrictions		
		P. corcovadensis	*P. niruri*	*P. tenellus*
2,4-Dichlorophenoxyacetic acid	0	42·0 ± 2·7	34·0 ± 2·5	40·0 ± 1·1
	3	–	–	25·0 ± 0·7**
	10	30·0 ± 2·0**	21·0 ± 2·4**	20·0 ± 0·4**
	30	21·0 ± 1·0**	13·0 ± 2·4**	20·0 ± 1·4**
	60	13·0 ± 1·5**	12·0 ± 1·9**	–
	90	15·6 ± 2·9**	–	–
ID50 (mg kg^{-1}) (and 95% confidence limits)		29·9 (22·5–39·8)	18·8 (11·4–31·4)	> 30·0
Maximal inhibitions (%)		69·0 ± 10·0	64·0 ± 9·0	53·0 ± 4·0
Indole-3-butyric acid	0	36·0 ± 0·5	–	37·1 ± 0·5
	3	–	–	26·0 ± 1·4**
	10	27·5 ± 1·4**	–	18·1 ± 1·7**
	30	19·7 ± 1·5**	–	19·3 ± 1·3**
	60	14·7 ± 0·4**	–	–
	90	14·0 ± 3·1**	–	–
ID50 (mg kg^{-1}) (and 95% confidence limits)		40·0 (28·0–59·0)	–	26·0 (18·0–38·0)
Maximal inhibitions (%)		60·0 ± 4·0	–	54·0 ± 11·0
Indole-3-acetic acid	0	–	–	37·1 ± 0·5
	3	–	–	26·0 ± 1·4**
	10	–	–	21·1 ± 0·7**
	30	–	–	22·4 ± 1·2**
ID50 (mg kg^{-1}) (and 95% confidence limits)		–	–	> 30·0
Maximal inhibitions (%)		–	–	48·0 ± 11·0

Each group represents the mean ± s.e.m. of six experiments. **$P < 0·01$ compared with the corresponding control value.

parison test. P values less than 0·05 were considered as indicative of significance. When appropriate, the ID50 (the dose of extracts of callus culture that reduced control responses by 50%) was estimated by graphical interpolation from individual experiments.

Results

In-vivo studies

Intraperitoneal injection of animals with the methanolic extract from callus culture of *P. corcovadensis*, *P. niruri* and *P. tenellus* medium, gave a dose-dependent inhibition of acetic

Table 2. Effect of the methanolic extract of callus culture of *Phyllanthus* given intraperitoneally against the first 0 to 5 min or the second phase, 15 to 30 min and paw oedema, in the mouse formalin test.

Methanolic extract *P. corcovadensis*[a]	Dose (mg kg^{-1})	Licking (s)		▲ Paw weight (mg)
		0–5 min	15–30 min	
	0	64·0 ± 1·6	152·0 ± 5·4	83·0 ± 2·5
	10	50·1 ± 1·3**	126·2 ± 8·2*	82·0 ± 5·8
	30	42·5 ± 2·0**	104·3 ± 7·0**	78·5 ± 3·9
	60	43·5 ± 2·8**	62·3 ± 5·8**	83·2 ± 7·6
	100	–	73·5 ± 4·9**	81·7 ± 5·6
ID50 (mg kg^{-1}) (and 95% confidence limits)		> 60	52·0 (42·0–65·0)	–
Maximal inhibition (%)		37·0 ± 9·0	63·0 ± 8·0	–
P. tenellus[a]	0	64·0 ± 1·6	147·8 ± 7·3	95·2 ± 4·5
	3	59·6 ± 2·5	–	–
	10	52·1 ± 3·1*	–	–
	30	53·8 ± 2·4	114·5 ± 5·4**	80·8 ± 3·9
	60	–	89·6 ± 8·3**	87·9 ± 3·0
	100	–	82·6 ± 6·0**	88·8 ± 3·5
ID50 (mg kg^{-1}) (and 95% confidence limits)		> 30	> 100	–
Maximal inhibition (%)		24·4 ± 7·7	49·0 ± 11·0	–
P. tenellus[b]	0	66·0 ± 1·9	160·6 ± 3·5	84·9 ± 5·6
	10	48·8 ± 3·7**	–	–
	30	33·5 ± 1·5**	148·0 ± 6·1	88·9 ± 5·9
	60	32·8 ± 1·1**	87·1 ± 3·3**	76·6 ± 2·6
	100	–	89·6 ± 7·2**	80·3 ± 5·8
ID50 (mg kg^{-1}) (and 95% confidence limits)		~ 60	~ 100	–
Maximal inhibition (%)		53·0 ± 6·0	49·0 ± 6·0	–

Each group represents the mean ± s.e.m. of six experiments. *$P < 0·05$, **$P < 0·01$ compared with the corresponding control value. Methanolic extract of callus culture obtained in either indole butyric acid[a] or indole acetic acid[b] medium.

Table 3. Effect of the ethyl acetate extract of callus culture of *P. corcovadensis* given intraperitoneally on acetic acid-induced abdominal constriction in mice.

Extract (mg kg^{-1})	Number of constrictions (mean ± s.e.m., n = 6)
0	39·1 ± 1·92
1	27·0 ± 2·36**
3	19·1 ± 1·90**

**$P < 0.01$ compared with corresponding control value.

acid-induced abdominal constriction response in mice (Table 1). Due to the limited availability of methanolic extracts of *P. corcovadensis* and *P. niruri* obtained in indole acetic acid medium, their analgesic activities could not be evaluated.

In the formalin test, the extract of callus of *P. tenellus* dose-dependently inhibited only the second phase of formalin-induced pain (Table 2). However, the extract did not interfere with formalin-induced paw oedema. Interestingly, the methanolic extract of callus culture obtained in indole acetic acid medium of *P. tenellus* caused a dose-dependent inhibition of both phases of formalin-induced nociception (Table 2). In addition, the extract from callus culture of *P. corcovadensis* was more active in inhibiting both phases of formalin-induced pain (Table 2). However, neither the extracts of *P. tenellus* nor those of *P. corcovadensis* (either indole acetic acid or indole butyric acid) interfered with formalin-induced paw oedema (Table 2).

The ethyl acetate extract obtained from the methanolic extract of callus culture of *P. corcovadensis* (1 and 3 mg kg^{-1}, i.p., 30 min before) caused a potent and graded inhibition of acetic acid-induced abdominal constriction response to mice (Table 3).

The analgesic effect of morphine (5 mg kg^{-1}, s.c.) but not that of *P. corcovadensis* (90 mg kg^{-1}, i.p.) callus culture extract obtained in indole butyric acid was fully prevented by naloxone (5 mg kg^{-1}, i.p.) (Fig. 1).

Tail-flick test

The methanolic extract of callus culture of *P. tenellus* (up to 50 mg kg^{-1}, i.p., 30 min before), obtained either in indole butyric acid or indole acetic acid medium, had no significant analgesic effect in the tail-flick test.

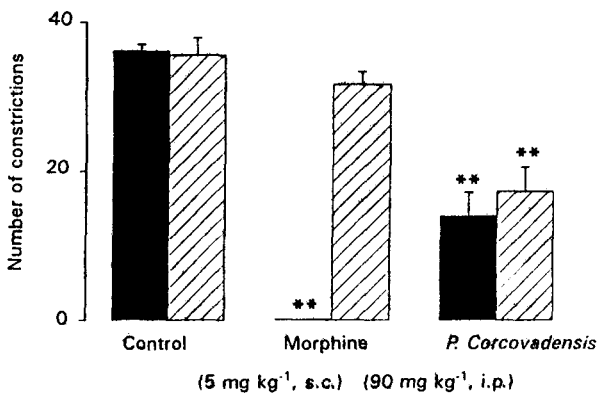

FIG. 1. Effect of naloxone on the antinociceptive profile caused by morphine (5 mg kg^{-1}, s.c.) or methanolic extract of *P. corcovadensis* (obtained in indole butyric acid medium) (90 mg kg^{-1}, i.p.) in the acetic acid-induced abdominal constrictions in mice. Each group represents the mean of 4 to 6 experiments and the vertical bars indicate the s.e.m. **$P < 0.01$ compared with corresponding control value. ■, Vehicle; ▨, naloxone 5 mg kg^{-1} i.p.

In-vitro studies

The methanolic extract from callus culture of *P. niruri*, *P. tenellus* and *P. corcovadensis* (20–320 μg mL^{-1}) did not cause any significant inhibition of the acetylcholine-induced contraction of the guinea-pig isolated ileum.

Discussion

We recently demonstrated that the hydroalcoholic extract obtained from the leaves, stems and roots of *Phyllanthus corcovadensis* exhibits a potent and long-lasting antinociceptive profile when analysed in several models of nociception in mice (Gorski et al 1993). The analgesic effect of the extract from *P. corcovadensis* appears to be unrelated to the inhibition of cyclo-oxygenase products derived from the arachidonic acid pathway, or to the stimulation of the opioid receptors, since the extracts failed to affect carrageenan-induced paw oedema and, in contrast to morphine, had no analgesic effect in the tail-flick model (Gorski et al 1993).

The results of the present study confirm and extend our previous findings, and clearly show that the potent systemic antinociceptive profile of the principle present in the leaves, stems and roots of *P. corcovadensis* is also present in in-vitro culture media. Furthermore, our data also demonstrate for the first time that the callus culture from other species of *Phyllanthus*, including *P. tenellus* and *P. niruri* possesses similar and potent analgesic properties. These results extend previous data reported in the literature (Unander 1991) which demonstrated that the aqueous extracts from callus culture of *P. amarus*, *P. abnormis* and *P. urinaria* obtained either in 2,4-dichlorophenoxyacetic acid or indole butyric acid, but not in indole acetic acid media, were effective in inhibiting hepatitis B virus DNA polymerase and reverse transcriptase, in-vitro.

In agreement with our previous study carried out with the hydroalcoholic extract of *P. corcovadensis* (Gorski et al 1993), the callus culture extracts from the three species of *Phyllanthus* analysed revealed similar and potent antinociceptive properties against acetic acid-induced abdominal constrictions and against the two phases of formalin-induced pain. However, the extract from callus culture was only one-tenth as potent as that reported for the hydroalcoholic extract of the *P. corcovadensis* plant. Furthermore, the efficacy of the methanolic extract from callus culture of *P. corcovadensis*, *P. niruri* and *P. tenellus* was reduced when compared with similar studies carried out with the hydroalcoholic extract of the leaves, stems and roots of *P. corcovadensis* (Gorski et al 1993). Interestingly, the indole acetic acid callus extract from *P. tenellus* was more effective then the indole butyric acid callus extract in inhibiting both phases of formalin-induced pain. To date we have no clear explanations for these findings. However, the higher efficacy of indole acetic acid medium could be related to the better control of synthesis of the active principle.

The mechanism that underlies the analgesic effect of the callus culture extracts of *Phyllanthus* species seems to be very similar to that recently reported for the extract of the

plant of *P. corcovadensis*. Thus, the extract of callus culture from *P. tenellus*, like the hydroalcoholic extract from *P. corcovadensis* (Gorski et al 1993), at a dose at which it markedly inhibited formalin or acetic acid-induced pain, failed to produce analgesia when tested in the tail-flick test. As reported previously, this nociceptive model is very sensitive to morphine but not to indomethacin (Gorski et al 1993). In addition, the analgesic effect caused by morphine but not that caused by callus of *P. corcovadensis* was completely prevented by naloxone, an opioid antagonist. These findings further reinforce our previous view (Gorski et al 1993), that the analgesic effect of extract of *Phyllanthus* species is not related to activation of opioid mechanisms. Preliminary unpublished results from our laboratory indicate that both prazosin and yohimbine, in conditions where they markedly prevented the analgesic effect induced by phenylephrine and clonidine, respectively, were ineffective in preventing the antinociceptive effect caused by the crude extract from *Phyllanthus*, suggesting that α-adrenoceptors seem not to be involved in their antinociceptive profile.

Another aspect investigated in the present study was whether the extract from callus culture of *Phyllanthus* species also interferes with agonist-induced contraction of the guinea-pig isolated ileum in-vitro. In contrast to that reported for the hydroalcoholic extract of *P. sellowianus* (Calixto et al 1984) or *P. niruri* (unpublished results), the extract of callus culture of *P. corcovadensis*, *P. tenellus* and *P. niruri*, up to $320\,\mu g\,mL^{-1}$, did not affect acetylcholine-induced contraction of the guinea-pig isolated ileum in-vitro. This suggests that the potent analgesic effect present in several species of *Phyllanthus* are unrelated to the reported antispasmodic activity.

A preliminary phytochemical analysis carried out with the extract of callus culture of *Phyllanthus* species, in the media used here, revealed that these extracts do not contain alkaloids nor flavonoids. However, HPLC analysis revealed the presence of seven compounds for *P. tenellus*, three for *P. corcovadensis* and five for *P. niruri*. Infrared and ^1H NMR analysis suggest that the main constituent present in the callus culture extracts of *P. tenellus* is a sugar, and also revealed the presence of phenolic compounds. Recently, Ishimaru et al (1992) demonstrated the presence of various phenolic constituents in tissue culture of *P. niruri*, including gallic acid, (−)-epicatechin, (+)-gallocatechin, (−)-epigallocatechin, (−)-epicatechin 3-*O*-gallate and (−)-epigallocatechin 3-*O*-gallate. Whether these compounds account for the antinociceptive actions by the callus culture extracts of the studied species of *Phyllanthus* reported here remains to be determined.

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