J. Pharm. Pharmacol. 1993, 45: 1046–1049
Received January 29, 1993

© 1993 J. Pharm. Pharmacol.

# Potent Antinociceptive Activity of a Hydroalcoholic Extract of *Phyllanthus corcovadensis*

FRANCO GORSKI, CLOVIS R. CORRÊA, VALDIR C. FILHO*, ROSENDO A. YUNES* AND JOÃO B. CALIXTO

*Department of Pharmacology and * Department of Chemistry, Universidade Federal de Santa Catarina, 88049 Florianópolis, SC, Brazil*

**Abstract**—This study analyses the analgesic effect of a hydroalcoholic extract (HE) from *Phyllanthus corcovadensis* in several models of pain in mice. HE (3–60 mg $kg^{-1}$, i.p.) or (100–500 mg $kg^{-1}$, p.o.) caused a graded and potent analgesic effect against the abdominal constriction response caused by acetic acid and acetylcholine with an ID50 of about 3 and 100 mg $kg^{-1}$, respectively. In the tail-flick model HE (up to 500 mg $kg^{-1}$, p.o.) was without effect, while morphine (1–10 mg $kg^{-1}$, s.c.) caused a graded increase in pain latency (ID50, 3 mg $kg^{-1}$). HE (1–300 mg $kg^{-1}$) given both intraperitoneally and orally caused a potent and graded inhibition of the second phase of formalin-induced persistent pain in mice with an ID50 of 1 and 80 mg $kg^{-1}$, respectively. In contrast, morphine (1–5 mg $kg^{-1}$, s.c.) inhibited both phases of formalin-induced pain with an ID50 of 2·5 mg $kg^{-1}$. Indomethacin (1–10 mg $kg^{-1}$, i.p.) only inhibited the second phase of formalin-induced pain with an ID50 of about 3 mg $kg^{-1}$. The analgesic effect of indomethacin, but not that caused by morphine and HE was accompanied by a graded inhibition of formalin-induced mouse paw oedema. In addition, HE up to 1 g $kg^{-1}$ failed to prevent carrageenan- and dextran-induced rat hindpaw oedema. It is concluded that HE exhibits a potent antinociceptive profile, either when given intraperitoneally or orally. The mechanisms that underly its analgesic effect are unclear at present, but appear to be unrelated to inhibition of synthesis of arachidonic acid via cyclo-oxygenase or to activation of opioid receptors.

The genus *Phyllanthus* (Euphorbiaceae) is composed of a great number of species that are widely distributed in many countries. In Brazil, the leaves, stems and roots of some species of this genus have been widely reported in folk medicine as traditional remedies for the treatment of kidney and bladder calculi. Infusions of *Phyllanthus* species have been popularly employed in many other countries for the treatment of diabetes, hepatitis, dysentery and against infections of the intestines (Morton 1981; Oliver-Bever 1983).

More recently, an increasing number of experimental and clinical studies, carried out with the extracts or with purified compounds from some plants of the genus *Phyllanthus*, have confirmed some of the most relevant medicinal uses of these plants reported in traditional medicine (Calixto et al 1984; Venkateswaran et al 1987; Thyagarajan et al 1988, 1990; Tempesta et al 1988; Blumberg et al 1989; Santos 1990; Unander 1991; Unander et al 1991; Shead et al 1992; for review see Unander et al 1990, 1992).

The purpose of the present study was to examine whether the hydroalcoholic extract (HE) from *P. corcovadensis* exhibits antinociceptive activity in several models of nociception in mice. In addition, we have evaluated the possibility of the antinociceptive activity of *P. corcovadensis* being secondary to an anti-inflammatory effect by comparing its actions against carrageenan- and dextran-induced rat hindpaw oedema.

## Materials and Methods

*Drugs*

Drugs used were: indomethacin, carrageenan $\lambda$ grade IV, dextran (Sigma Chemical, USA), morphine (Merck). All reagents used were high grade purity. Indomethacin was prepared in 0·9% w/v NaCl containing 5% $NaHCO_3$. All other drugs were dissolved in 0·9% w/v NaCl or in physiological buffer solution (PBS).

Correspondence: J. B. Calixto, Department of Pharmacology, Universidade Federal de Santa Catarina, Rua Ferreira Lima 82, 88015420, Florianópolis SC, Brazil.

*Preparation of the crude extract*

The dried leaves, stems and roots of *P. corcovadensis* were minced and extracted with 50% ethanol-water in the proportion of 1:3 (w/v), stirred mechanically at room temperature (21 °C) for 24 h. The ethanol was evaporated and the extract was concentrated to the desired concentration.

*Antinociceptive activity*

*Acetic acid-induced abdominal constriction.* Male Swiss mice, 25–30 g, were kept in a temperature controlled environment (23 °C) with a 12 h light-dark cycle. Food and water were freely available. Abdominal constrictions to acetic acid (0·6%, i.p.) or acetylcholine (6 mg $kg^{-1}$, i.p.) were monitored as described previously (Collier et al 1968; Bentley et al 1981). The animals were pretreated with HE intraperitoneally (3–60 mg $kg^{-1}$) 30 min beforehand or orally (100–500 mg $kg^{-1}$) 60 min before injection of irritants. HE was dissolved in a physiological buffer solution. Control animals received a similar volume of buffer solution (10 mL $kg^{-1}$). All experiments were carried out at 20–22 °C. After challenge, pairs of mice were placed in separate boxes and the number of constrictions were cumulatively counted over a period of 20 min for acetic acid and 10 min for acetylcholine. Analgesic activity was expressed as the inhibition of the number of constrictions between control animals and mice pretreated with HE.

*Formalin-induced peripheral pain.* Male Swiss mice, 20–30 g, were injected under the dorsal surface of the skin in one hindpaw with 20 $\mu$L of 0·5% formalin solution, made up in

Table 1. Effect of the hydroalcoholic extract of *Phyllanthus corcovadensis* given intraperitoneally or orally on acetic acid-induced abdominal constrictions mice.

| HE (mg kg$^{-1}$) | Number of constrictions |
|---|---|
| Intraperitoneal | |
| 0 | 43±4 |
| 3 | 19±2** |
| 10 | 10±2** |
| 30 | 3±2** |
| 60 | 1±2** |
| Oral | |
| 0 | 44±2 |
| 100 | 24±2** |
| 300 | 18±2** |
| 500 | 19±2** |

Each point represents the mean±s.e.m. of six experiments. ** $P<0.01$ compared with control (Dunnett's test).

Table 2. Effect of the hydroalcoholic extract of *Phyllanthus corcovadensis* given intraperitoneally or orally on acetylcholine-induced abdominal constrictions in mice.

| HE (mg kg$^{-1}$) | Number of constrictions |
|---|---|
| Intraperitoneal | |
| 0 | 7·7±0·7 |
| 3 | 4·8±0·6** |
| 10 | 1·8±0·4** |
| 30 | 0·2±0·1** |
| Oral | |
| 0 | 9·1±1·2 |
| 100 | 7·5±1·7 |
| 200 | 6·0±0·8* |
| 300 | 6·1±0·8* |

Each point represents the mean±s.e.m. of 6–8 experiments. * $P<0.05$, ** $P<0.01$ compared with control (Dunnett's test).

physiological buffer. Two mice (control and treated) were observed simultaneously for 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. Nociceptive scores normally peaked about 5 min after formalin injection (first phase) and 20 to 30 min after formalin injection (second phase), representing the tonic pain response; this is accompanied by an inflammatory response and the release of algesic mediators. Animals were treated with 50–300 mg kg$^{-1}$ HE orally or 1–30 mg kg$^{-1}$ intraperitoneally, 60 and 30 min before injection of formalin, respectively. Indomethacin (1–10 mg kg$^{-1}$, i.p.) and morphine (1–5 mg kg$^{-1}$, s.c.) were given 30 and 60 min before formalin injection, respectively, and were used as positive controls. A separate group of mice received only the vehicle (10 mL kg$^{-1}$) used to dilute HE or drugs.

*Tail-flick test.* Male Swiss mice, 25–30 g, were used. A radiant-heat tail-flick analgesiometer was used to measure response latencies, according to the procedure described previously by D'Amour & Smith (1941) with minor modifications. Animals responded to a focused-heat-stimulus by flicking or removing their tail, exposing a photocell located in the apparatus immediately below the tail. The reaction time was recorded for control mice or for animals pretreated with HE or morphine.

An automatic 8 s cut-off was used to prevent tissue damage. Each animal was selected on the basis of its reactivity in the model. On the test day, each subject was given four base-line tail-flick tests before drug or vehicle injection. A latency period of 20 s was defined as complete analysis. HE was administered orally (up to 500 mg kg$^{-1}$) 60

Table 3. Effect of morphine and the extract of *Phyllanthus corcovadensis* on the tail-flick test.

| Morphine (mg kg$^{-1}$) | Latency (s) |
|---|---|
| 0 | 4·3±1·2 |
| 1 | 5·4±0·9 |
| 3 | 10·0±1·1** |
| 10 | 19·4±0·6** |
| HE concn (mg kg$^{-1}$) | |
| 0 | 5·7±1·4 |
| 500 | 4·9±1·6 |

Each group represents the mean±s.e.m. of 6–8 experiments. ** $P<0.01$ compared with control (Dunnett's test).

Table 4. Effect of the hydroalcoholic extract of *Phyllanthus corcovadensis* given either intraperitoneally or orally against the first 0 to 5 min or the second phase, 20 to 30 min, in the mouse formalin test.

| HE (mg kg$^{-1}$) | Licking (s) | |
|---|---|---|
| Intraperitoneal | 0–5 min | 20–30 min |
| 0 | 63±3 | 72±7 |
| 1 | — | 36±5** |
| 3 | 53±5 | 18±8** |
| 10 | 51±7 | 14±5** |
| 30 | 41±6* | 2±2** |
| Oral | | |
| 0 | 50±4 | 70±5 |
| 50 | 44±3 | 43±4** |
| 100 | 45±5 | 33±5** |
| 200 | 52±6 | 17±6** |
| 300 | 42±8 | 8±5** |

Each group represents the mean±s.e.m. of 6–8 experiments. * $P<0.05$, ** $P<0.01$ compared with control (Dunnett's test).

Table 5. Effect of indomethacin and morphine against the first 0 to 5 min and the second phase, 20 to 30 min in the mouse formalin test.

| Indomethacin (mg kg$^{-1}$) | Licking (s) | |
|---|---|---|
| | 0–5 min | 20–30 min |
| 0 | 63·5±5 | 106±6 |
| 1 | 61·0±4 | 75±5** |
| 3 | 56·5±6 | 60±5** |
| 10 | 70·0±5 | 27±6** |
| Morphine (mg kg$^{-1}$) | | |
| 0 | 66±6 | 100±6 |
| 1 | 39±3** | 68±4** |
| 2·5 | 22±2** | 44±3** |
| 5·0 | 8±1** | 2±1** |

Each group represents the mean±s.e.m. of 6–8 experiments. ** $P<0.01$ compared with control (Dunnett's test).

Table 6. Effect of indomethacin and the hydroalcoholic extract from *Phyllanthus corcovadensis* on formalin-induced paw oedema in mice.

| | Dose (mg kg$^{-1}$) | Increase in paw weight (mg) | Inhibition (%) |
|---|---|---|---|
| Saline (i.p.) | — | 65±2 | — |
| Indomethacin (i.p.) | 1 | 66±5 | 0 |
| | 3 | 56±4 | 14* |
| | 10 | 47±3 | 26** |
| *P. corcovadensis* (i.p.) | 3 | 64±5 | 1 |
| | 10 | 57±3 | 12 |
| | 30 | 60±3 | 6 |
| Saline (p.o.) | | 62±2 | 0 |
| *P. corcovadensis* (p.o.) | 50 | 54±5 | 13 |
| | 100 | 65±6 | 0 |
| | 200 | 59±5 | 6 |

Each group represents the mean of 6–8 animals. *$P<0.05$; **$P<0.01$ (Dunnett's test).

min before experiments. Control animals received the same volume of vehicle.

*Anti-inflammatory activity. Rat paw oedema.* Male rats, 130–180 g, were pretreated with different doses of HE (0·5 and 1 g kg$^{-1}$) orally 1 h before receiving an intraplantar injection of 0·1 mL of either carrageenan (300 μg/paw) or dextran (300 μg/paw) into hindpaws. The contralateral paw received an equal volume of PBS and was used as control. The oedematogenic response was evaluated by the use of a plethysmometer at 0·5, 1, 2 and 4 h after intraplantar injection of the phlogistic agents. Oedema was reported in mL as the difference between the value of the irritant-injected and the saline-injected paw.

*Statistical analysis*

The results are presented as the mean±s.e.m. and were analysed by analysis of variance followed by Dunnett's test or by unpaired Student's *t*-test, as appropriate. *P* values less than 0·05 were considered as significant. When appropriate, the ID50 (i.e. the dose of HE or drugs that reduced the response by 50%) was estimated by graphical interpolation from individual experiments.

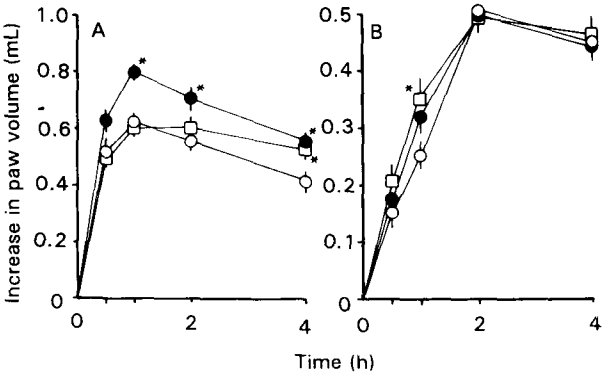


FIG. 1. Effect of hydroalcoholic extract of *Phyllanthus corcovadensis* against dextran (A) and carrageenan (B)-induced rat hindpaw oedema. Each point represents the mean of six experiments and the vertical lines indicate the s.e.m. ○ Control, ● 0·5 g kg$^{-1}$, □ 1·0 g kg$^{-1}$. *$P<0.05$ compared with control (unpaired *t*-test).

## Results

Tables 1, 2 show that HE (3–60 mg kg$^{-1}$) caused a graded and potent analgesic effect against abdominal constrictions elicited by acetic acid and acetylcholine. The calculated ID50 was about 3 mg kg$^{-1}$ and the maximal inhibition of the nociceptive response was 100%. Given orally, HE (100–500 mg kg$^{-1}$) also caused a significant inhibition against acetic acid- and acetylcholine-induced constrictions.

In the tail-flick model, HE (500 mg kg$^{-1}$, p.o.) was virtually ineffective as an analgesic agent, while morphine (1–10 mg kg$^{-1}$, s.c.) caused a graded increase in pain latency with an ID50 of 3 mg kg$^{-1}$ (Table 3).

Table 4 shows that HE (1–30 mg kg$^{-1}$, i.p.) caused a marked and dose-dependent inhibition of formalin-induced peripheral pain. However, its analgesic effect was more pronounced against the second phase of the pain model, with an ID50 of about 1 mg kg$^{-1}$ and maximal inhibition of 100%. Given orally, HE (50–300 mg kg$^{-1}$, 60 min before) caused dose-dependent inhibition of the second phase of formalin-induced pain in mice, with an ID50 of about 80 mg kg$^{-1}$ and maximal inhibition of 90% (Table 4). In the same experimental conditions, morphine (1–5 mg kg$^{-1}$, s.c.), inhibited in a graded manner and with similar potency both the first and the second phases of formalin-induced pain (ID50 2·5 mg kg$^{-1}$) (Table 5). In contrast, indomethacin (1–10 mg kg$^{-1}$, i.p.) was effective in antagonizing only the second phase of formalin-induced pain (ID50 3 mg kg$^{-1}$, and maximal inhibition 80%) (Table 5). The analgesic effect of indomethacin, but not that caused by HE and morphine, was accompanied by a significant inhibition of formalin-induced paw oedema (Table 6). Similarly, HE (0·5 and 1 mg kg$^{-1}$, p.o.) failed to prevent both dextran- and carrageenan-induced rat hindpaw oedema but caused a slight, but significant, increase of hindpaw oedema induced by either agent (Fig. 1).

## Discussion

The results of the present study show that HE obtained from the leaves, stems and roots of *P. corcovadensis*, given either intraperitoneally or orally, caused a potent antinociceptive effect in mice. Of particular interest were the results demonstrating that HE, given either intraperitoneally or orally, was extremely active in antagonizing the second phase of the persistent pain produced by peripheral administration of formalin in mice. These results strongly support the view that the active principle present in HE is much more effective in attenuating hyperalgesic or persistent pain, than the neurogenic pain caused by formalin. It has been widely reported that formalin-induced persistent pain in mice paw involves two distinct phases; a neurogenic pain which corresponds to the first phase, followed by an inflammatory pain that is accompanied by release of inflammatory mediators, designated as second phase (Hunskaar et al 1985, 1986; Abbott & Franklin 1986; Hunskaar & Hole 1987; Murray et al 1988). In addition, it has been postulated that formalin-mediated peripheral pain may be analogous to human postoperative pain (Abbott & Franklin 1986).

The antinociceptive potency of HE is comparable or even more potent than that reported for indomethacin (Dray & Dickenson 1991; Moore et al 1991). However, in marked contrast with indomethacin, HE failed to inhibit formalin-induced- as well as carrageenan- and dextran-induced rat hindpaw oedema. These results indicate that HE produces analgesia by a mechanism independent of inhibition of the synthesis of cyclo-oxygenase products derived from arachidonic acid.

The antinociceptive activity of HE also appears to occur by mechanisms independent of activation of opioid receptors, since morphine was found to be very effective in antagonizing both phases of formalin-induced peripheral pain, but there was a complete lack of analgesic effect of HE against radiant heat in the tail-flick test conditions where morphine caused a graded increase in paw latency (ED50 3 mg $kg^{-1}$).

Concerning the chemical constituents responsible for the potent analgesic effect of HE from *P. corcovadensis*, recent unpublished results from our laboratories show the existence of at least two active principles in this plant that may account for this effect. However, this plant contains many other constituents that may also contribute to its analgesic activity.

The results of the present study demonstrate that HE of the leaves, stems and roots of *P. corcovadensis* exhibits potent antinociceptive activity when given either intraperitoneally or orally to mice. The mechanisms underlying its analgesic effect remain unknown to some degree; however, they seem to be unrelated to either the inhibition of the synthesis of cyclo-oxygenase products of arachidonic acid or to activation of opioid receptors. Chemical and pharmacological studies are underway in order to characterize the constituents responsible for the analgesic effect and also to furnish additional evidence concerning their mechanisms of action.

*Acknowledgements*

The authors are indebted to Dr Pedro Melillo Magalhães, Universidade de Campinas, Campinas SP, who collected and classified this plant. This study was supported by grants from Conselho Nacional de Desenvolvimento Cientifico e Tecnológico and by Financiadora de Estudos e Projetos, Brazil.

## References

Abbott, F. V., Franklin, K. B. J. (1986) Noncompetitive antagonism of morphine analgesia by diazepam in the formalin test. Pharmacol. Biochem. Behav. 24: 319–321

Bentley, G. A., Newton, S. H., Starr, J. (1981) Evidence for an action of morphine and enkephalin on sensory nerve endings in the mouse peritoneum. Br. J. Pharmacol. 73: 325–333

Blumberg, B. S., Millman, I., Venkateswaran, P. S., Thyagarajan, S. P. (1989) Hepatitis B virus and hepatocellular carcinoma-treatment of HBV carriers with *Phyllantus amarus*. Cancer Detect. Prevent. 1: 195–201

Calixto, J. B., Yunes, R. A., Neto, A. S. O., Valle, R. M. R., Rae, G. A. (1984) Antispasmodic effects of an alkaloid extracted from *Phyllanthus sellowianus*: a comparative study with papaverine. Braz. J. Med. Biol. Res. 17: 313–321

Collier, H. O. J., Dinnen, L. C., Johnson, C. A., Schneider, C. (1968) The abdominal constriction response and its suppression by analgesic drugs in the mouse. Br. J. Pharmacol. 32: 295–310

D'Amour, F. E., Smith, J. (1941) A method for determining loss of pain sensation. J. Pharmacol. 72: 74–79

Dray, A., Dickenson, A. (1991) Systemic capsaicin and olvanil reduce the acute algogenic and the late inflammatory phase following formalin injection into rodent paw. Pain: 78–83

Hunskaar, S., Hole, K. (1987) The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain 30: 103–114

Hunskaar, S., Fasmar, O. B., Hole, K. (1985) Formalin test in mice: a useful technique for evaluating mild analgesics. J. Neurosci. Methods 14: 69–76

Hunskaar, S., Berge, O. G., Hole, K. (1986) Dissociation between antinociceptive and anti-inflammatory effects of acetylsalicyclic acid and indomethacin in the formalin test. Pain 25: 125–132

Moore, P. K., Oluyomi, A. O., Babbedge, R. C., Wallace, P., Hart, S. L. (1991) L-$N^G$-nitro arginine methyl ester exhibits antinociceptive activity in mouse. Br. J. Pharmacol. 102: 198–202

Morton, J. F. (1981) In: Thomas C. C. (ed.), Atlas of Medicinal Plants in Middle America. 1st ed. Springfield, pp 458–462

Murray, C. W., Porreca, F., Cowan, A. (1988) Methodological refinements in the mouse paw formalin test - an animal model of tonic pain. J. Pharmacol. Methods 20: 175–186

Oliver-Bever, B. (1983) Medicinal plants in tropical West Africa III. Anti-infection therapy with higher plants. J. Ethnopharmacol. 9: 1–83

Santos, D. R. dos. (1990) Chá de Quebra-pedra (*Phyllanthus niruri*) na Litíase Urinária em Humanos e Ratos. PhD Thesis, Escola Paulista de Medicina, São Paulo, p. 157

Shead, A., Vickery, K., Pajkos, A., Medhurst, R., Freiman, J., Dixon, R., Cossart, Y. (1992) Effects of *Phyllanthus* plant extracts on duck hepatitis B in vitro and in vivo. Antiviral Res. 18: 127–138

Tempesta, M. S., Corley, D. G., Beutler, J. A., Metral, C. J., Yunes, R. A., Giacomozzi, C. A., Calixto, J. B. (1988) Phyllanthimide, a new alkaloid from *Phyllanthus sellowianus*. J. Natur. Prod. 3: 617–618

Thyagarajan, S. P., Subramanian, S., Thirunalasundari, T., Venkateswaran, P. S., Blumberg, B. S. (1988) Effect of *Phyllanthus amarus* on chronic carriers of hepatitis B virus. Lancet ii: 764–766

Thyagarajan, S. P., Jayaram, S., Valliammai, T., Madanagopalan, N., Pal, V. G., Jayaraman, K. (1990) *Phyllanthus amarus* and hepatitis B. Lancet ii: 949–950

Unander, D. W. (1991) Callus induction in *Phyllanthus* species and inhibition of viral DNA polymerase and reverse transcriptase by callus extracts. Plant Cell Reports 10: 461–466

Unander, D. W., Webster, G. L., Blumberg, B. S. (1990) Records of usage or assays in *Phyllanthus* (Euphorbiaceae) I. Subugenera *Isocladus*, *Kirganelia*, *Cicca* and *Emblica*. J. Ethnopharmacol. 30: 233–264

Unander, D. W., Webster, G. L., Blumberg, B. S. (1991) Uses and bioassays in *Phyllanthus* (Euphorbiaceae): a compilation II. The subgenus *Phyllantus*. J. Ethnopharmacol. 34: 97–133

Unander, D. W., Webster, G. L., Blumberg, B. S. (1992) Usage and bioassays in *Phyllanthus* (Euphorbiaceae): a compilation III. The subgenera *Erioccocus*, *Conami*, *Gomphidium*, *Botryanthys*, *Xylophylla* and *Phyllanthodendron*, a complete list of the species cited in the three-part series. J. Ethnopharmacol. 36: 103–112

Venkateswaran, P. S., Millman, I., Blumberg, B. S. (1987) Effects of an extract from *Phyllanthus niruri* on hepatitis B and woodchuck hepatitis viruses: in vivo and in vitro studies. Proc. Natl. Acad. Sci. USA 84: 274–278