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Chemical and Pharmacological Studies of *Phyllanthus caroliniensis* in Mice

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Abstract

The aim of this study was to isolate and characterize the constituents of the hydroalcoholic extract (HE) of the leaves, stems and roots from *P. caroliniensis*, and also to evaluate the preliminary antinociceptive action of the HE and purified compounds in mice.

Phytosterols, quercetin, gallic acid ethyl ester and geraniin were identified in *P. caroliniensis* on the basis of ^{1}H and ^{13}C NMR spectral data and by mixed co-TLC and co-HPLC injection with authentic samples. The HE of *P. caroliniensis* (10–100 mg kg^{-1}, i.p.) inhibited, in a dose-related manner, acetic acid-induced abdominal constrictions in mice, with a mean ID50 value of 23·7 mg kg^{-1}. In the formalin test, the HE given intraperitoneally (1–30 mg kg^{-1}) or orally (25–600 mg kg^{-1}) caused graded inhibitions of both the neurogenic (first phase) and the inflammatory response (late phase) of formalin-induced licking. The HE was 54-fold more effective in inhibiting the late phase than it was in inhibiting the first phase of the formalin test, with mean ID50 values of 3·6 and 196·4 mg kg^{-1}, respectively. The HE failed, however, to affect the oedematogenic response associated with the late phase of formalin-induced pain. In addition, the reference drug, aspirin, given intraperitoneally (1–100 mg kg^{-1}) or orally (100–600 mg kg^{-1}), caused significant inhibition of the late but not the first phase of the formalin test. Pharmacological analysis also revealed that quercetin, gallic acid ethyl ester and a semi-purified fraction of flavonoids (1–100 mg kg^{-1}, i.p.) exhibited graded and significant antinociception against acetic acid-induced abdominal constriction. The mean ID50 values (mg kg^{-1}) for these effects were: 18·8, 34·7 and 5·3, respectively.

It is concluded that quercetin, gallic acid ethyl ester and some as yet unidentified flavonoids might account for the antinociceptive action reported for the HE of *P. caroliniensis*.

Previous studies conducted by our group have demonstrated that the hydroalcoholic extract (HE) obtained from the leaves, stems and roots of several plants belonging to the genus *Phyllanthus* (Euphorbiaceae), such as *P. corcovadensis, P. niruri, P. urinaria, P. tenellus, P. sellowianus*, and the callus culture obtained from some plants of this genus, caused dose-related antinociception in mice (Gorski et al 1993; Santos et al 1994, 1995a, b). In contrast to that reported for the non-steroidal antiinflammatory drugs, these HEs were also effective in preventing the neurogenic response caused by formalin or capsaicin (Santos et al 1995b). The mechanism underlying the antinociception caused by the HEs of these plants still remains unclear, but is unlikely to be associated with interaction of opioid, adrenergic (either α_1 or α_2) or serotonergic systems, nor did the HEs interfere with the L-arginine nitric oxide pathway (Santos et al 1995a, b). When assessed in-vitro the HE of *P. urinaria* caused concentration-dependent contraction of guinea pig trachea and guinea pig urinary bladder (Dias et al 1995; Paulino et al 1996). The contraction caused by HE of *P. urinaria* in both preparations depends largely on extracellular calcium influx insensitive to both nicardipine and $^{\omega}$conotoxin, selective antagonists of L- and N-type calcium channels, respectively. In the guinea pig trachea at least, however, the contraction caused by the HE of *P. urinaria* seems to involve an interaction with the neurokinin system, through the NK_1 and NK_2 receptors (Paulino et al 1996). The active principles responsible for such actions are not yet completely known, but different classes of compounds such as steroids, tannins and flavonoids, among others, have been isolated from these plants and might be responsible, at least in part, for these actions (Santos et al 1995c; Miguel et al 1995a, b).

We now report the isolation and chemical identification of some phenolic constituents from the leaves, stems and roots of another species of *Phyllanthus, P. caroliniensis*. We have also assessed the preliminary antinociceptive effect, against acetic acid and formalin-induced nociception, of the HE, of two pure compounds isolated from this plant, quercetin and gallic acid methyl ester, and a mixture of unidentified flavonoids isolated from *P. caroliniensis*.

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Material and Methods

Plant material and spectroscopy

Botanical material of *P. caroliniensis* was collected in July 1993 in Urussanga, State of Santa Catarina, Brazil, and was classified by Dra. Leila da Graça Amaral and Ms Mirian Ulyssea (Department of Botany, Universidade Federal de Santa Catarina, Brazil). A voucher specimen (ref. 22740) was deposited in the herbarium FLOR of the Department of Botany, UFSC, Florianpolis, Brazil.

IR spectra were recorded on a Perkin Elmer PC FTIR apparatus. ^{1}H NMR (200 and 300 MHz) and ^{13}C NMR (50 and 75 MHz) spectra were obtained on Brucker 200 and Varian XL 300 devices, respectively, and chemical shifts were measured from TMS as an internal standard. Detection of eluates was achieved by HPTLC (Merck) and chromatograms were revealed using $FeCl_3$ reagent for flavonoids and tannins and sulphuric anisaldehyde for sterols and terpenes, as described previously (Ikan 1976).

Drugs

The drugs used were acetic acid, formalin, Tween 80 (Merck, Darmstadt, Germany), aspirin (Sigma, St Louis, MO, USA). All other reagents used were of a high grade of purity. Semi-purified fraction and pure compounds of *P. caroliniensis* were dissolved in absolute ethanol (10 mg mL^{-1}) and diluted just before use with 0·9% NaCl solution. Aspirin was dissolved in 0·5% Tween 80. The HE was dissolved in 0·9% NaCl solution. The final concentrations of ethanol and Tween 80 did not exceed 5%, levels which had no effect on the results.

Chemical extraction and fractionation

The hydroalcoholic extract obtained from dried leaves, stems and roots of *P. caroliniensis* (200 g) was successively partitioned with hexane, dichloromethane and ethyl acetate to furnish the semi-purified extracts (0·02 g, 0·1 g and 1·06 g, respectively). The ethyl acetate fraction was chromatographed on a silica gel (40 g) column eluted with a $CHCl_3$-MeOH gradient (100:0, 95:5, 90:10, 85:15, 80:20, 70:30, 50:50 and 0:100), 100 mL each, yielding gallic acid ethyl ester (28 mg) and quercetin (66 mg), respectively. The residue (616 mg) was re-chromatographed over Sephadex LH 20 (1 g), eluted with methanol-water 70:30 (100 mL), yielding a fraction containing a mixture of two flavonoids (184 mg), which was named FF, and an ellagitannin identified as being geraniin (124 mg). All compounds obtained were compared with authentic samples using co-TLC and co-HPLC injection. The spectroscopic data for geraniin was in agreement with that previously described (Okuda et al 1982). Chromatography of the hexane extract, performed in a similar manner to the procedures previously reported for *P. corcovadensis* (Santos et al 1995c) revealed the presence of small quantities of a mixture of phytosterols (β-sitosterol, stigmasterol and campesterol); the compounds were characterized by CG-MS.

Abdominal constriction response caused by intraperitoneal injection of dilute acetic acid

Male Swiss mice, 25–30 g, were kept in a temperature-controlled environment (23 ± 2°C) with a 12-h light-dark cycle. Food and water were freely available. The abdominal constriction was induced by intraperitoneal injection of acetic acid (0·6%) according to the procedures described previously (Santos et al 1994), and consisted of a contraction of the abdominal muscle and a stretching of the hind limbs. Animals were pre-treated intraperitoneally (1-100 mg kg^{-1}, 30 min before) or orally (25–400 mg kg^{-1}, 60 min before) with the HE, semi-purified fraction (FF), quercetin or gallic acid ethyl ester, obtained from *P. caroliniensis* before injection of acetic acid. Control animals received the same volume of 0.9% NaCl solution (10 mL kg^{-1}). All experiments were performed 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 between control animals and mice pre-treated with HE, pure compounds or semi-purified fractions.

Formalin-induced licking

Male Swiss mice, 25–30 g, were used. The procedure was similar to that described previously (Corrêa & Calixto 1993; Santos et al 1995a). Briefly, 20 µL 2·5% formalin (0·92% formaldehyde) made up in phosphate-buffer solution was injected under the surface of the right hind paw. Two mice (control and treated) were observed simultaneously from 0 to 30 min after formalin injection. The amount of time spent licking the injected paw was timed with a chronometer and was considered as indicative of nociception. The initial nociceptive scores normally peaked 5 min after formalin injection (first phase) and 15–30 min after formalin injection (second phase), representing the tonic and inflammatory pain responses, respectively (Hunskaar & Hole 1987). Animals were treated with aspirin or with the HE of *P. caroliniensis* intraperitoneally (1–100 mg kg^{-1}) or orally (50–600 mg kg^{-1}), 30 and 60 min before formalin injection, respectively. Control animals received only the vehicle (10 mL kg^{-1}) used to dilute the HE and aspirin. After intraplantar injection of formalin, the animals were immediately placed into a glass cylinder 20 cm in diameter, and the time spent licking the injected paw was determined. At the end of all experiments the animals were killed by cervical dislocation and the paws were cut at the knee and weighed.

Statistical analysis

The results are presented as mean ± s.e.m., and statistical significance between groups was determined by analysis of variance then by Dunnett's multiple comparison test. P values less than 0·05 ($P < 0·05$) were considered as indicative of significance. When appropriate, the mean ID50 values (i.e. the dose of HE, semi-purified fractions, pure compounds or aspirin which reduced responses by 50% relative to control values) were estimated by graphical interpolation from individual experiments.

Results

The intraperitoneal administration to animals of HE of *P. caroliniensis* (10–90 mg kg^{-1}), 30 min before injection of acetic acid, produced dose-related and significant inhibition of acetic acid-induced abdominal constrictions. The calculated mean ID50 value was 23·7 mg kg^{-1}. Given orally up to 400 mg kg^{-1} 1 h before experiments, the HE failed to inhibit acetic acid-induced nociception (Table 1). When tested in the formalin model, the HE of *P. caroliniensis* administered intraperitoneally (1–30 mg kg^{-1}) or orally (50-600 mg kg^{-1}) caused significant and dose-related inhibition of both the neurogenic (early phase) and the inflammatory (late phase) of formalin-induced licking (Table 2). The HE was, however, approximately 3- to 10-fold more active in preventing the late phase of the formalin-induced licking, depending on the route of administration used, but it was 54-fold more potent when given intraperitoneally than when given orally (Table 2). The HE did not, however, significantly change the paw oedema

Table 1. Effect of hydroalcoholic extracts of *P. caroliniensis* on acetic acid-induced abdominal constriction in mice.

Treatment	Number of abdominal constrictions			
	Dose (mg kg^{-1})	Intraperitoneal	Dose (mg kg^{-1})	Oral
P. caroliniensis	0	34·0 ± 1·0	0	33·4 ± 0·5
	10	26·0 ± 1·0*	50	29·8 ± 2·7
	30	14·5 ± 0·7*	100	34·0 ± 1·5
	60	10·3 ± 1·6*	200	33·6 ± 1·7
	90	11·6 ± 2·0*	400	29·0 ± 2·6
ID50†		23·7 (19·7–28·5)		> 400
Maximum inhibition (%)		70·0 ± 5·0		12·0 ± 7·0

†ID50 (mg kg^{-1}) with 95% confidence limits. Each group represents the mean ± s.e.m. of 6 to 8 animals. *$P < 0.01$ compared with respective control values.

Table 2. Effect of hydroalcoholic extracts of *P. caroliniensis* against the first phase, 0 to 5 min, or the second phase, 15 to 30 min, of the formalin test on mice.

Hydroalcoholic extract	Dose (mg kg^{-1})	Licking (s) 0–5 min	15–30 min	Δ Paw weight (mg)
Intraperitoneal	0	66·4 ± 2·8	160·2 ± 9·6	68·3 ± 1·4
	1	65·5 ± 4·3	165·7 ± 10·4	63·8 ± 4·5
	3	49·2 ± 3·8*	86·8 ± 8·1*	74·5 ± 5·9
	10	38·0 ± 3·1*	21·4 ± 9·9*	66·1 ± 4·5
	30	37·7 ± 4·3*	21·8 ± 9·8*	65·0 ± 6·6
ID50†		> 30	3·6 (2·3–5·6)	
Maximum inhibition (%)		44·0 ± 6·0	87·0 ± 6·0	
Oral	0	63·5 ± 2·9	209·4 ± 12·5	71·2 ± 3·3
	50	49·6 ± 1·9*	161·6 ± 11·5**	65·6 ± 4·2
	100	43·3 ± 1·6*	137·0 ± 15·2*	64·9 ± 4·9
	200	42·3 ± 1·2*	94·4 ± 11·9*	66·5 ± 3·5
	400	43·0 ± 3·1*	64·5 ± 14·0*	59·1 ± 2·5
	600	43·5 ± 3·8*	63·0 ± 15·5*	66·5 ± 2·9
ID50†		> 600	196·4 (168·7–228·6)	
Maximum inhibition (%)		33·0 ± 2·0	70·0 ± 7·5	

†ID50 (mg kg^{-1}) with 95% confidence limits. Each group represents the mean ± s.e.m. of 6 to 14 animals. *$P < 0.01$, **$P < 0.05$ compared with respective control values.

Table 3. Effect of aspirin against the first phase, 0 to 5 min, or the second phase, 15 to 30 min, in the formalin test on mice.

Aspirin	Dose (mg kg^{-1})	Licking (s) 0–5 min	15–30 min	Δ Paw weight (mg)
Intraperitoneal	0	84·4 ± 2·0	116·2 ± 3·9	44·0 ± 6·1
	10	69·8 ± 2·0	71·2 ± 4·0*	40·0 ± 4·9
	30	71·1 ± 0·9	63·3 ± 3·3*	40·0 ± 3·0
	60	69·8 ± 2·5	41·7 ± 8·2*	44·0 ± 3·6
	100	—	14·5 ± 2·6*	48·6 ± 2·6
ID50†		> 60	22·1 (13·8–37·6)	
Maximum inhibition (%)		17·3 ± 2·0	88·0 ± 3·0	
Oral	0	65·4 ± 3·8	155·3 ± 7·3	76·8 ± 4·7
	100	65·8 ± 4·9	138·1 ± 5·4	70·9 ± 2·5
	200	59·9 ± 4·3	99·3 ± 8·3*	65·4 ± 5·0
	400	60·1 ± 2·6	52·1 ± 11·2*	57·6 ± 3·2**
	600	—	14·4 ± 2·6*	56·7 ± 3·8**
ID50†		> 400	282·0 (243·0–328·0)	
Maximum inhibition (%)		8·5 ± 1·6	93·0 ± 3·0	

†ID50 (mg kg^{-1}) with 95% confidence limits. Each group represents the mean ± s.e.m. of 6 to 8 animals. *$P < 0.01$, **$P < 0.05$ compared with respective control values.

associated with the late phase of the formalin-induced licking (Table 2). The reference drug aspirin, given intraperitoneally (10–100 mg kg^{-1}) or orally (100–600 mg kg^{-1}), caused significant inhibition against the late but not the first phase of the formalin test (Table 3). The calculated mean ID50 values were 22·1 and 282·0 mg kg^{-1}, respectively. When aspirin was given

Table 4. Effect of the quercetin, gallic acid ethyl ester and flavonoid fraction on acetic acid-induced abdominal constriction in mice.

Compound	Number of abdominal constrictions			
	Dose (mg kg^{-1})	Intraperitoneal	Dose (mg kg^{-1})	Oral
Quercetin	0	35·8 ± 1·5	0	45·9 ± 2·0
	3	22·6 ± 1·9*	50	42·8 ± 2·6
	10	10·0 ± 2·4*	100	34·2 ± 1·7*
	30	2·3 ± 0·5*	200	35·4 ± 0·6*
ID50†		18·8 (15·7–22·6)		> 200
Maximum inhibition (%)		88·0 ± 1·0		26·0 ± 1·4
Gallic acid ethyl ester	0	45·8 ± 3·6	—	—
	10	37·2 ± 1·9	—	—
	30	23·8 ± 4·5*	—	—
	60	16·2 ± 1·9*	—	—
	100	5·8 ± 0·9*	—	—
ID50†		34·7 (27·5–43·7)		
Maximum inhibition (%)		88·0 ± 2·0		
Flavonoid fraction	0	44·8 ± 2·9	—	—
	1	35·0 ± 1·7	—	—
	3	17·7 ± 4·1*	—	—
	10	9·5 ± 2·5*	—	—
	30	4·3 ± 1·6*	—	—
ID50†		5·3 (3·8–7·5)		
Maximum inhibition (%)		90·0 ± 4·0		

†ID50 (mg kg^{-1}) with their respective 95% confidence limits. Each group represents the mean ± s.e.m. of 6 to 10 animals. *$P < 0.01$ compared with respective control values.

orally, however, it produced partial but significant inhibition of the oedematogenic response associated with the late phase of the formalin test (Table 3).

The chemical partition of the HE and the chromatographic procedures used enabled the isolation of several compounds; these were identified as quercetin, gallic acid ethyl ester, geraniin and three phytosterols, stigmasterol, β-sitosterol and campesterol. A flavonoid fraction was also isolated, the components of which have not so far been identified. When assessed in the abdominal constriction induced by acetic acid, both quercetin and gallic acid ethyl ester (3–100 mg kg^{-1}, i.p.), given 30 min before acetic acid, caused dose-related and significant antinociception with mean ID50 values of 18·8 and 34·7 mg kg^{-1}, respectively (Table 4). Given orally 1 h before acetic acid, quercetin (100 and 200 mg kg^{-1}) also caused partial though significant inhibition of acetic acid-induced abdominal constriction (Table 4). Similarly, the semi-purified flavonoid fraction (1–30 mg kg^{-1}, i.p.), given 30 min before acetic acid, produced dose-related inhibition of the acetic acid-induced abdominal constriction, with a mean ID50 value of 5·3 mg kg^{-1} (Table 4). Because of the limited quantity of gallic acid ethyl ester and the flavonoid fraction, it was not possible to test them orally against acetic acid-induced abdominal constriction.

Discussion

The genus *Phyllanthus* (Euphorbiaceae) comprises more than 600 species which are widely distributed throughout tropical and subtropical countries (Unander et al 1995). *P. caroliniensis* is a native plant of Brazil and the infusion of its leaves, stems and roots has been used in traditional medicine as a diuretic (Morton 1981). So far, to the best of our knowledge, chemical and pharmacological studies have not been performed on this plant.

The results of our studies have demonstrated, as we have reported previously for the HE for other species of plants from the genus *Phyllanthus* such as *P. niruri, P. urinaria, P. corcovadensis, P. tenellus* and *P. sellowianus* (Santos et al 1995a, b), that the HE of *P. caroliniensis* caused dose-related antinociception when assessed against acetic acid and against the neurogenic (early phase) and the inflammatory (late phase) of formalin-induced licking. Similar to that reported for other species of *Phyllanthus*, the HE of *P. caroliniensis* was much more effective in preventing the inflammation than it was at preventing the neurogenic response induced by formalin. In addition, the HE was several times less potent and also less efficacious when given orally. Very similar results have been reported for other *Phyllanthus* species (Santos et al 1995a, b). Compared with a standard drug (aspirin), the HE of *P. caroliniensis* was about 2 to 6-fold more active in causing antinociception, depending on the route of administration used. In contrast with non-steroidal antiinflammatory drugs (Gorski et al 1993 and this study), the active principle(s) present in the HE of *P. caroliniensis*, as reported previously for other species of *Phyllanthus*, were also quite effective in attenuating the neurogenic component of formalin-induced licking.

The chemical studies performed with *P. caroliniensis* enabled the isolation and identification of phytosterols and some phenolic compounds, such as the flavonoid quercetin, the phenolic compounds gallic acid ethyl ester and geraniin, and also a mixture of unidentified flavonoids. All compounds were

characterized by direct comparison with authentic samples and also, usually, by comparison of spectroscopic data (Okuda et al 1982; Miguel et al 1996). Gallic acid ethyl ester has been reported in other species of plants belonging to the genus *Phyllanthus*, e.g. *P. emblica* (Nag & Khanna 1973) and *P. sellowianus* (Miguel et al 1995a).

The ellagitannin geraniin has been reported in several species of *Phyllanthus*, e.g. *P. niruri* (Ueno et al 1988), *P. urinaria* (Okuda et al 1980), *P. amarus* (Foo 1993) and *P. sellowianus* (Miguel et al 1995a, 1996). Besides its analgesic effect (Miguel et al 1996), this compound has the ability to reduce systemic blood pressure by inhibition of noradrenaline release (Cheng et al 1994). Geraniin also inhibits the formation of 5-lipoxygenase and cyclooxygenase products derived from the arachidonic-acid pathway in rat peritoneal polymorphonuclear leukocytes (Kimura et al 1986).

We characterized geraniin by analysis of its complex ^{1}H and ^{13}C NMR spectra, which revealed the presence of doubling signals, attributed to an equilibrium five-to-six member hemiacetal structures of the didehydrohexahydroxydiphenoyl groups in solution. We also confirmed the structures of the geraniin by synthesis of its derivative phenazine A, obtained by condensation of geraniin with *O*-phenyllediamine; the physicochemical and spectroscopic data of this derivative were identical with those described in the literature (Okuda et al 1982; Foo 1993).

Quercetin is a common flavonoid present in many plants; it has been isolated from several species of *Phyllanthus* (Gupta & Ahmed 1984; Miguel et al 1995a). In attempts to identify the flavonoids present in FF, we performed several TLC separations of this fraction and compared the chromatograms with those obtained from common flavonoids previously isolated from other species of plant belonging to the genus *Phyllanthus*, e.g. rutin and quercetrin (Nara et al 1977; Miguel et al 1995a; Gupta & Ahmed 1984). The R_F values obtained for the two flavonoids of FF were quite different to those of the standards, indicating that the flavonoids present in *P. caroliniensis* were chemically different. All compounds isolated from *P. caroliniensis* have already been isolated from other species of *Phyllanthus*, e.g. *P. corcovadensis* and *P. sellowianus* (Santos et al 1995c; Miguel et al 1995a, b, 1996). Our results seem to indicate that some compounds isolated from *P. caroliniensis*, e.g. the phytosterols, quercetin, gallic acid ethyl ester, geraniin and the flavonoid mixture, contribute to the antinociception reported for the HE of *P. caroliniensis* and other species of *Phyllanthus* (Gorski et al 1993; Santos et al 1995a). We have recently reported the antinociceptive effect of geraniin isolated from *P. sellowianus*; this was shown to be approximately eight times more active than acetaminophen and aspirin when tested against abdominal constriction induced by acetic acid (Miguel et al 1996). Taken together, our current and previous findings (Santos et al 1995a, b; Miguel et al 1995a, b, 1996) strongly support the view that different classes of constituents, such as steroids, flavonoids and tannins, can account for the antinociceptive action reported for the HE obtained from the leaves, stems and roots of several species of plant belonging to the genus *Phyllanthus*, including *P. caroliniensis*. Chemical and pharmacological studies are currently in progress to determine the nature of the constituents present in the FF, and also to identify other active compounds present in the HE of *P. caroliniensis*.

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