Neuropharmacological Activity of Extracts from *Passiflora incarnata*

E. Speroni[1,3] and A. Minghetti[2]

Received: February 17, 1988

Abstract: A fluid extract of *Passiflora incarnata* L. was studied for its neuropharmacological properties. This extract, orally and intraperitoneally administered to rats, raised the nociceptive threshold in the tail-flick and hot-plate tests, but not in the vocalization test. On intraperitoneal injection, the extract significantly prolonged sleeping time, protected animals from the convulsive effect of pentylenetetrazole, and affected locomotor activity. The fluid extract was partitioned by solvents and chromatography and at least two products, one lipophilic (A) and one hydrophilic (B), were found to be active. They could not be ascribed to either alkaloids or the flavonoid structures present in the extract.

Introduction

Passiflora incarnata has long been used for its sedative action (1, 2). It is still employed against sleeplessness and seems also to have an antineuralgic effect (3). Little is known about the mechanism(s) of action of *P. incarnata* extracts. A few *P. incarnata* species have been chemically investigated and products such as flavonoids (3, 4, 5), β-carboline alkaloids (6, 7, 8, 9), cyanogenic glycosides (10), and terpene derivatives (11, 12, 13) have been identified. Nevertheless, the active principles responsible for the neuropharmacological properties have still not been recognized. The present study was designed to isolate the active products.

Materials and Methods

Instrumentation

UV spectra were recorded using a Bausch and Lomb Spectronic 2000 spectrophotometer. For HPLC analyses, a Beckman chromatograph equipped with two pumps, model 110, and unset wavelength detector model 165 were used. Separations were made in isocratic conditions using LiChrosorb RP-8 columns 25 × 0.4 cm with MeCN/phosphate buffer pH 8 (60/40) as solvent system, at a flow rate of 1 ml/min.

Chemicals

The following compounds were obtained from the sources indicated: harman, Fluka; pentobarbital and pentylenetetrazole from Sigma; naloxone from Endo Lab., Garden City, N.Y.; and vitexine and isovitexine from Inverni della Beffa, Milan (Italy).

Animals

Male Sprague-Dawley rats (200–250 g) and Swiss mice (25–30 g) were used, housed under standard environmental conditions (room temperature 20 ± 2 °C, humidity 60 %), according to GLP procedures.

Plant material

An ethanolic extract of *P. incarnata* L., was obtained from Aboca Erbe (Sansepolcro – Arezzo, Italy), and prepared according to the French Pharmacopoeia (Ed. VIII). It was made from one-year-old plants of which only the aereal parts were employed. Plants were harvested in July 1986 and extracted as soon as harvested. The identity of *P. incarnata* was confirmed by the Botany Institute of the University of Perugia, Italy.

Preparation of the extracts

The ethanolic extract from leaves and stems of *P. incarnata* (fluid extract) having a water content of about 50 %, was evaporated under reduced pressure at a temperature below 45 °C to about one quarter of the initial volume in order to get rid of the ethanol. Then water was added to reach half the volume of the fluid extract at the start. The resulting solution was considered as the "aqueous extract". Its pH was 5.6 and it was left unchanged throughout extraction procedures. The content in solid material after lyophilization was 40.3 mg/ml.

The aqueous extract was used for pharmacological tests, either intact or freeze-dried, and diluted as necessary with sterile saline solution for either oral or intraperitoneal administration.

The aqueous solution was extracted first with an equal volume of petroleum ether (b.p. 40–70 °C) three times and the pooled extracts were dried over Na_2SO_4 and evaporated under reduced pressure. The resulting solid was considered Fraction I.

The aqueous layer was extracted again as above with *n*-BuOH saturated with H_2O. The pooled butanolic extracts were reduced to one tenth of the volume by distillation under reduced pressure, then diluted with H_2O and freeze-dried (Fraction II). The exhausted aqueous phase was also lyophilized (Fraction III).

For pharmacological tests, Fraction I was dissolved in ethanol and part was diluted with at least nine volumes of sterile saline solution and sonicated. Control solutions containing up to 10 % ethanol were ineffective in the pharmacological tests employed. Lyophilized Fractions II and III were dissolved in sterile saline solution and sonicated as above to obtain a complete solution.

Chromatographic procedures

Fraction I was analysed by TLC on Merck silica gel G plates using CH_2Cl_2 as solvent system (system a). Fraction II was analysed by TLC on RP-8 plates in the solvent system H_2O-EtOH (80 : 20) (system b).

In order to detect the biological activity on the plates, segments of 1 cm each were scraped off and eluted with ethanol. The solvent was filtered through a Millex HV (Millipore) filter, evaporated under nitrogen, and the residue resuspended with a few drops of dimethylformamide in sterile saline solution and administered to the animals.

Harmane was determined by HPLC using a solution of 1 μg/ml in CH_3CN as reference. A quantity corresponding to 0.1 μg was added to 2 ml of concentrated aqueous extract corresponding to 10 ml of fluid ex-

[1] Instituto di Farmacologia, Via Irnerio, 48, Facoltá di Farmacia, Universitá di Bologna, I-40126 Bologna, Italy.
[2] Dipartimento di Scienze Farmaceutiche, Via Belmeloro, 6, Facoltá di Farmacia, Universitá di Bologna, I-40126 Bologna, Italy.
[3] Address for correspondence.

tract of *P. incarnata*. It was brought to pH 9 with diluted ammonia, adsorbed on a 3 ml Extrelut column, and eluted with 30 ml of CH_2Cl_2. The eluted solvent was evaporated, the residue dissolved in ml 1 CH_3CN and aliquots were injected in to the HPLC. More than 85 % of the added harman was recovered. an identical sample of 2 ml of aqueous extract, without addition of harman, was processed similarly.

Administration of plant material

Animals were treated with extracts as reported in the preparation of plant material. Doses and routes are set out in Table I.

Table I. Partitioning of 100 ml of aqueous extract of *P. incarnata* and pharmacological activity of each fraction.

	Starting material	Fractions		
		I	II	III
Content of each fraction (mg)		12	246	1756
Percent of the total extract	100	0.6	11.1	88.3
Dose (mg/kg. *i.p.*)	160	4.0	30.0	224.0
Flavonoids content (mg)*	4.5	0.0	5.5	1.7
Pharmacological activity**	+	+	+	−

* Content of vitexine and isovitexine present in the doses administered.

** The pharmacological activity was evaluated for all three tests reported in Materials and Methods.

Analgesimetric procedures

The nociceptive threshold was evaluated using the tail flick (14), the hot plate (15), and the vocalization (16) tests. In the tail flick test, the strength of the radiant heat was adjusted so as to obtain a tail flick latency of 3.7 ± 0.15 s in control animals and values were recorded by an automated device; a cut-off time of 10 s was fixed. For the hot plate test, a plate temperature of 53 ± 0.5 °C was fixed. The maximum time allowed for an animal to respond was 30 s. For the vocalization threshold (measured in mA), two stainless steel 30 gauge electrodes were inserted in the middle section of the tail. Electrical stimulation was applied of 1 s duration, containing 125 shocks of 1.6 ms width delivered from a high frequency square wave constant current generator. The maximal intensity of the current delivered was 2 mA.

Individual baseline tail flick, hot plate latency, and vocalization thresholds were determined in three pre-tests 20 min before drug administration.

Convulsive threshold

The onset of convulsive episodes in 2–3 s after pentylenetetrazole (50 mg/kg *i.p.*) and subsequent death in controls or animals pretreated with 160 mg/kg of aqueous extract were evaluated.

Pentobarbital-induced sleeping time

The time elapsed between loss and recovery of the righting reflex was taken as the sleeping parameter and recorded (17) for control and drug-pretreated animals. Ten minutes after administration of *P. incarnata* aqueous extract (160 mg/kg *i.p.*), mice were given an intraperitioneal dose of 50 mg/kg of pentobarbital.

Motility test

Spontaneous locomotor activity was recorded using an activity cage (U. Basile, Milano) with automatic counting of the animals' movements across the bars on the cage floor (18). Naive mice were placed in activity cages for 30 min before injection and recording of activity. Motor activity was recorded for 120 min after treatment.

Acute toxicity

The lyophilized aqueous extract dissolved in sterile saline solution was injected intraperitoneally to mice and its toxicity was observed for 48 hours.

Statistics

For statistical analysis Student's t test was used.

Results

Extraction of 100 ml of the fluid extract of *P. incarnata* with solvents of increasing polarity as reported in Materials and Methods gave the results shown in Table I. For the biological activity of the extracts, each sample was assayed in all three tests reported in Materials and Methods. Fraction I extracted with petroleum ether contained only 12 mg corresponding to 0.6 % of the solid material present in the fluid extract. Dissolved in 2.5 ml of absolute ethanol and 0.5 ml, diluted ten-fold with saline solution, showed biological activity on mice and rat at the dose of 4.0 mg/kg (see Table I). When the dosage was halved, the activity both in mice and rats was negligible or absent.

The ethanolic solution of Fraction I is pale yellow and its UV and visible spectra have the most evident peaks at 475 and 450 nm with in addition high absorbance below 250 nm. No vitexine or flavonoids are present in this fraction.

Fraction II extracted with *n*-BuOH, and lyophilized, was a gray powder corresponding in weight to 11.1 % of the starting material. Dissolved in saline solution it showed biological activity in mice and rats at a dose of 30 mg/kg. A methanolic solution of fraction II gave a UV spectrum with peaks at 340 and 270 nm with the shape and changes at different pH values characteristic of flavonoid derivatives. Its content in flavonoids, expressed as vitexine, corresponded to 18.2 % of its dry weight.

Fraction III, the aqueous layer exhausted after combined extractions with petroleum and *n*-butanol, contained nearly 90 % of the solid starting material and when lyophilized looked like a brownish powder. Dissolved in saline solution it had no biological activity in mice even at a dose of 224 mg/kg. Its UV spectrum had peaks at 320 and 270 nm and its total content in flavonoid derivatives corresponded to 0.82 % of its dry weight.

In order to assess whether the biological activity of *P. incarnata* was due to a single product that could be extracted in the experimental conditions in petroleum ether and *n*-butanol or to two or more different products, chromatographic studies were made.

Fraction I was chromatographed on silica gel plates in solvent system a). Each segment of the plate was tested and activity was recovered in the segment between R_f 0.6 and 0.8. In this region only traces of absorbance with UV light at 254 nm were evident. Evidence of a product was obtained only on exposing the plate to iodine vapours.

One of the products in *P. incarnata* with pharmacological activity reported in the literature and probably present in fraction I is harman (6). However when tested by TLC in system a), harman showed an $R_f = 0.05$ very distant from the active product. In addition, when determined by HPLC as reported in Materials and Methods, harman did not exceed 0.01 µg/ml in the aqueous extracts. Considering that pharmacological activity of aqueous extracts in mice is evident at 160 mg/kg, the amount of

harman present in the dose corresponded to the negligible amount of 40 ng/kg. In addition, harman administered to mice at 4 mg/kg had no sedative action. All these data rule out the harman as being responsible for the pharmacological activity observed.

In order to verify whether other alkaloids were present in Fraction I, a TLC plate in system a) was sprayed with Dragendorff's reagent, but only a faint colour appeared near the starting line.

Fraction II was also chromatographed in order to isolate the active product. In chromatographic system b) activity was found at R_f from 0.85 to 0.95. In this region UV absorbance is very low and the colour on exposure to iodine vapours was very faint compared with the three evident spots of flavonoid derivatives present at R_f ranging from 0.0 and 0.3, the main of which were vitexine and isovitexine and the minor one covered the 10% of the total flavonoids. These flavonoids were inactive when scraped from the TLC plates, determined by UV absorbance and tested in animals, each at the doses of 5.0 mg/kg. In addition, pure vitexine and isovitexine tested at the doses of 5.0 and 10.0 mg/kg in mice, showed no activity.

Effects of P. incarnata extract on nociceptive threshold

As shown in Fig. 1, *P. incarnata* aqueous extracts given to rats at oral doses of 160 mg/kg raised the threshold to nociceptive stimuli of radiant heat in the tail flick test (A) and hot plate heat (B), but not of electrical stimulation (data not reported). In the first test considered (tail flick) 30 min after *P. incarnata* extract administration, there was a statistically significative increase of the latency to reaction reaching a maximal value within 180 min. After this peak there was a gradual decrease within the 300 min.

In the second test (hot plate), the reaction time to the nociceptive stimulus was modified 30 min from administration and remained at the maximal value throughout the observation period for the upper dose. The response declined more slowly than the tail flick reaction times. There was no changes in the threshold to a nociceptive stimulus for the vocalization test.

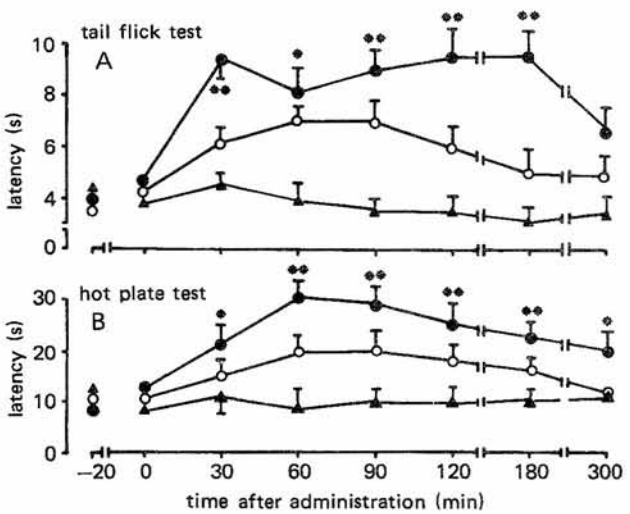

Fig. 1. Effects on nociceptive threshold of a *P. incarnata* extract given intraperitoneally to rats: tail flick latency (panel A), hot plate latency (panel B).
(▲——▲) vehicle, (●——●) *P. incarnata* extract, 160 mg/kg *i.p.*, (○——○) *P. incarnata* extract, 80 mg/kg *i.p.* M ± SEM n = 10.
* $p < 0.05$ vs. control, ** $p < 0.01$ vs. control.

Effect on convulsive threshold

P. incarnata extract significantly increased the onset time of convulsions and prolonged survival in mice after pentylenetetrazole injection.

Effect on sleeping time

The sleeping time induced by pentobarbital was prolonged by *P. incarnata* extract (Fig. 2) and by Fraction I and II but not by Fraction III.

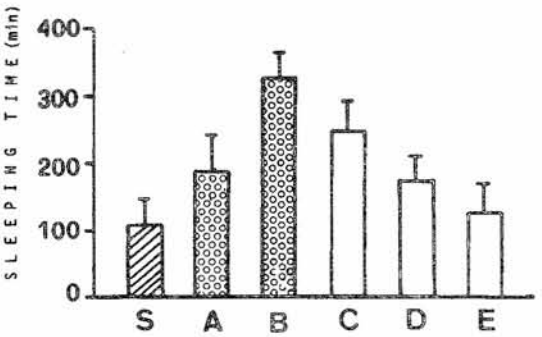

Fig. 2. Effect of a *P. incarnata* extract on pentobarbital-induced sleeping time in mice after injection of *P. incarnata* extract or its fractions.
S = saline; A = *P. incarnata* extract, 80 mg/kg; B = *P. incarnata* extract, 160 mg/kg; C = Fraction I, 10 mg/kg; D = Fraction II, 30 mg/kg; E = Fraction III, 240 mg/kg.
Each bar indicates minutes elapsed between loss and recovery of righting reflex. M ± SEM n = 10.
** $p < 0.01$ vs. control.

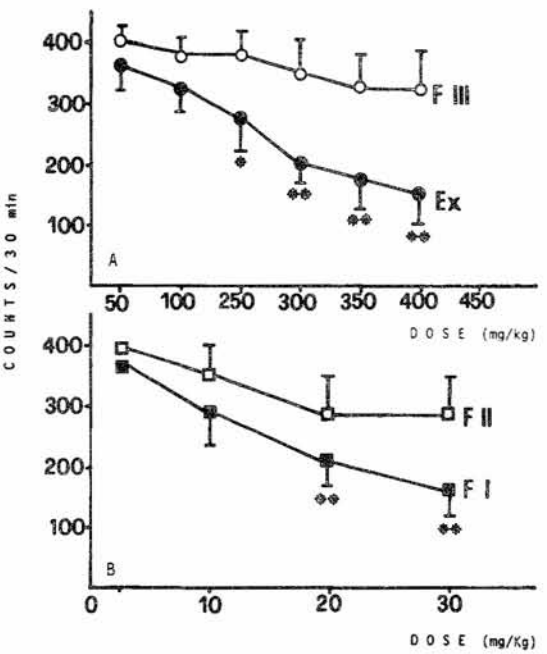

Fig. 3. Effect of different doses of *P. incarnata* extract and its fractions on locomotor activity in mice. Recording of locomotor activity was started 30 min after *i.p.* administration over a period of 30 min. (■——■) F I, (□——□) F II, (○——○) F III, (●——●) *P. incarnata* extract (Ex). M ± SEM, n = 10, * $p < 0.05$.

Motility test

The effects on locomotor activity, recorded for *P. incarnata* extract and its fractions, are illustrated in Fig. 3.

Acute toxicity

P. incarnata extract had an acute toxicity value higher than 900 mg/kg in mice.

Discussion

It appears from this study that *P. incarnata* extract has a complex activity on the CNS. The findings of the antinociception experiments support the old hypothesis about *P. incarnata's* antinociceptive activity, even if it is effective only for some analgesimetric procedures, as reported for another plant extract: *Cannabis sativa* L. (19).

The rise in the nociceptive threshold could be due to motor impairment induced by the *P. incarnata* extract. Locomotor activity was in fact significantly affected by the extract, as was the sleeping time induced by pentobarbital.

The present findings on *P. incarnata* central effect suggest the need for further investigations to evaluate possible relationships with CNS neurotransmitters.

From a chemical point of view, the biological activity of *P. incarnata* fluid extracts is due to at least two different products. One (product A) behaves like a very lipophilic compound. Its chemical class does not seem to correspond either to aromatic or heterocyclic structures in view of its low absorbance in the UV region.

The other active product (product B) present in Fraction II, in contrast with product A, behaves like a very polar compound. It seems to have the same chemical characteristics as A. Fraction II contains the great majority of the flavonoid derivatives of the *P. incarnata* fluid extract, but these compounds can be ruled out as responsible for the activity.

It can be assumed that the same active structure is present in products A and B, A being the aglycone and B its hydroxylated or glycosylated derivative. Studies are now in progress to elucidate their structures.

References

(1) Fellow, E. J., Smith, C. S. (1938) J. Am. Chem. Soc. 27, 565–573.
(2) Ruggy, G. H., Smith, C. S. (1940) J. Am. Chem. Soc. 29, 207–208.
(3) Neu, R. (1954) Arzneim.-Forsch. 4, 292–295 and 601–605.
(4) Neugebauer, H. (1949) Pharmazie 4, 176–180.
(5) Ulubelen, A., Oksuz, S. (1982) J. Nat. Prod. 45, 783–784.
(6) Bennati, E. (1971) Boll. Chim. Farm. 110, 664–669.
(7) Lutomski, J., Malek, B. (1975) Planta Med. 27, 222–225.
(8) Bennati, E., Fedeli, E. (1968) Boll. Chim. Farm. 107, 716–720.
(9) Lutomski, J., Malek, B., Rybacka, L. (1975) Planta Med. 27, 112–121.
(10) Spencer, K., Seigler, D. S. (1985) Phytochemistry 24, 2615–2617.
(11) Andreetti, G. D., Bocelli, G., Sgarabotto, P. (1977) J. Chem. Soc. Perkin Trans. 2, 605–609.
(12) Orsini, F., Verotta, L. (1985) J. Chrom. 349, 69–75.
(13) Orsini, F., Pellizzoni, F., Verotta, L. (1986) Phytochemistry 25, 191–193.
(14) D'Amour, F. E., Smith, D. L. (1941) J. Pharmacol. Exp. Ther. 72, 74–81.
(15) Nathan, B. E., Leimbach, D. (1953) J. Pharm. Exp. Ther. 107, 385–390.
(16) Paalzow, G., Paalzov, L. (1975) Acta Pharm. Toxicol. 32, 22–32.
(17) Ferrini, R., Miragoli, G., Taccardi, B. (1974) Azrneim.-Forsch. 24, 2029–2032.
(18) Hutchins, D. A., Rogers, K. J. (1971) Br. J. Pharmac. 43, 504–513.
(19) Ferri, S., Cavicchini, E., Romualdi, P., Speroni, E., Murari, G. (1986) Psychopharmacology 89, 244–247.