Vasoconstrictor Activity of 8-O-Acetylharpagide from Ajuga reptans

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VASOCONSTRICTOR ACTIVITY OF 8-*O*-ACETYLHARPAGIDE FROM *AJUGA REPTANS*

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ABSTRACT.—The traditional therapeutic indications for the use of *Ajuga reptans* (Labiatae) have been investigated. The H_2O-soluble part of a crude and partially purified MeOH extract and two isolated iridoids (8-*O*-acetylharpagide and harpagide), were tested for a biological activity on isolated smooth muscle preparations from guinea pig.

Ajuga reptans L. (Labiatae), well known from ancient time as a vulnerary drug (1–4), is reported in the literature also with a mild narcotic, antirheumatic (5), astringent, and tonic (4) activity, as well as with an antifeedant effect on insects (6). It is still used in the Tuscany folk medicine as an antihemorrhagic, vulnerary, and cicatrizant agent (7). The folk use is not supported by experimental evidence, although other species of the same genus show a contractile activity on guinea pig myometrium (8) and a blood-clotting action in the rabbit (9). Consequently, it was of some interest to investigate the activity of the crude extracts and of the pure components, first in preliminary experiments on intestinal musculature and then on isolated vasal preparations, with the aim of identifying the active principles. For this purpose the pharmacological study was performed in parallel to a phytochemical research on the active constituents of the plant, in which terpenoids (10,11), sterols (12), phytoecdysones (13), and iridoids (14–16) have been previously isolated.

Morpho-anatomic and histochemical investigations on glandular hairs (site of synthesis and accumulation of secondary products in Labiatae) were, at the same time, carried out with the aim of localizing the isolated constituents.

EXPERIMENTAL

PLANT MATERIAL.—Plants were collected in late spring 1989 in the wild near Seravezza and Stazzema (Apuan Alps, northwest Tuscany). Dried specimens of the plant are deposited in the Herbarium of Pisa University (PI).

BOTANICAL ANALYSIS.—Sections 20–30 μm thick, obtained by a Leitz Kriostat 1720 D from fresh material and leaf strips, were used for histochemical and anatomical analysis. They were stained with common histochemical reagents for lipophilic substances (17), essential oils (18), and pectin-like substances (17,19). Since histochemical methods for iridoids are not available, the reactions with the Komarowsky reagent (20) were tested on the sections and on the leaf strips, using lower temperatures and concentrations in order to avoid tissue destruction, at increasing contact times.

CHEMICAL ANALYSIS.—Melting points were determined with a Kofler apparatus and are uncorrected. Nmr spectra were recorded in CD_3OD/D_2O with an 80 MHz Varian CFT–20 and a 200 MHz Bruker AC 200 P spectrophotometers, using TMS as internal standard. Optical rotations were determined in MeOH solutions (1 mg/ml) at 23° with a Perkin-Elmer 241 polarimeter. Tlc analysis was performed on Si gel 60 F_{254} Merck precoated plates [eluent $CHCl_3$-MeOH-H_2O (6:4:1)]. The spots were visualized at 254 nm or by spraying with 4-hydroxybenzaldehyde/H_2SO_4 (Komarowsky reagent) and heating at 105° for 3–4 minutes.

EXTRACTION.—Fresh aerial parts (600 g) of *A. reptans*, deprived of the inflorescence, were extracted with MeOH, in the dark, for 20 days at room temperature by mean of an agitator. The

solution was completely evaporated under reduced pressure at a temperature below 45°, obtaining 39.12 g (6.59%) of residue (MR). An MeOH solution of MR (1.0 g), after extraction with $CHCl_3$ to eliminate chlorophyll, gave a positive Trim-Hill test for iridoids. A portion of MR (2.0 g), dissolved in H_2O (PV), after filtration, was used for preliminary pharmacological tests.

Isolation of iridoids.—The residue MR (2.0 g), dissolved in H_2O, was chromatographed over an Amberlite IRC–50H$^+$ column (Fluka, 32–45 mesh, 2.5×40 cm). Elution with H_2O and EtOH gave an aqueous (I), a hydroalcoholic (II), and an alcoholic (III) fraction. The last two fractions were identical by tlc and were active in pharmacological tests. In order to obtain a sufficient amount of active substances, 25.0 g of MR residue was solubilized in MeOH-H_2O (1:4) and extracted with $CHCl_3$ and *n*-BuOH in that order. The last fraction, completely evaporated under reduced pressure, dissolved in MeOH and treated with Et_2O, gave a residue R (7.3 g) which was chromatographed on an Amberlite XAD–2 column (Fluka, 20–50 mesh, 400 g of resin/g residue) using H_2O and MeOH as eluents. Usual workup gave an aqueous, a hydroalcoholic, and an alcoholic fraction. The latter two, as a consequence of tlc analysis, were combined and chromatographed on a Si gel 60 column (Merck, 70–230 mesh; 1×20 cm) using $CHCl_3$-MeOH-H_2O (6:4:1) as eluent and collecting 48 fractions (3 ml) which were analyzed by tlc. Fractions 6–12 (0.0063 g) of the Si gel chromatography gave pure 8-*O*-acetylharpagide (mp 156–158°, $[\alpha]^{23}D$ −154°, R_f 0.52). Fractions 15–24 (0.017 g) gave pure harpagide (mp 153–155°, $[\alpha]^{23}D$ −132°, R_f 0.40). The ^1H- and ^{13}C-nmr spectral data are in good agreement with those of the literature (21).

PHARMACOLOGICAL ANALYSIS.—*Animals.*—Male guinea pigs, weighing 300–350 g, were used. The animals were sacrificed by cervical dislocation and bled by excision of the jugular veins. The animals used for intestinal preparations were deprived of food intake for 24 h before the experiments.

Isolated guinea pig ileum.—The abdominal cavity was opened by a midline excision, and a segment of the ileum (length 2–3 cm), located a few cm above the ileum-caecal valve, was removed. After careful cleaning of the surrounding mesenteric tissue the two open ends were tied with inextensible thread by means of a surgical needle. The preparation was attached to the organ holder and to the recording system, suspended in a 10 ml organ bath, and submitted to a tension of 1.0 g.

Isolated guinea pig colon.—The terminal colon (length 2–3 cm) was withdrawn and prepared like the ileum, both for the cleaning up and for the assembling of the preparation.

Isolated guinea pig aorta.—After opening the thorax, the descending thoracic aorta was taken away, cleaned of the connective tissue, and cut spirally, obtaining a ribbon 2 mm in width and 25 mm in length. The aortic strips were set up under a 2 g tension in a 10 ml organ bath.

The bathing of the organs in vitro was performed with Tyrode solution of the following composition (mmol/liter): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.9, NaH_2PO_4 0.3, $NaHCO_3$ 11.9, and glucose 5.6. They were maintained at 37° and gassed with a mixture of O_2 (95%) and CO_2 (5%).

The organs were left to stabilize for 60 min before beginning the experiments. All the fractions and the isolated iridoids were dissolved in distillated H_2O or Tyrode solution after evaporation of the solvents. When an EtOH extract was assayed (only in the ileal preparation), a blank test was performed with the same amount of 95% EtOH to exclude any activity of the solvent. Concentrations are expressed as g of solid (extract or iridoid or physiologic agonist) in final solution volume.

Physiologic agonists were used as reference drugs. The complete dose-response curve to physiologic agonists was obtained by recording the contractions or relaxations to increasing concentrations of the agonist with the technique of cumulative doses. The same procedure was repeated for the crude extract and the other examined fractions. The cumulative volume added to the bath was of 0.3 ml. When it was necessary to add a greater volume, the extracts or the iridoid were dissolved directly in Tyrode solution, and an equal volume was drawn out from the bathing fluid.

Recording.—Basal tone and responses to drugs were recorded by means of an isotonic lever Basile (model 7006), connected to a microdynamometer Basile (model 7050) with a magnification of 10 for aortic strips and 3–4 for intestinal segments. In order to evaluate the nature of the agonism some experiments were carried out in the presence of phentolamine, a non-selective α-antagonist.

Drugs.—The substances used were norepinephrine bitartrate (Sigma), acetylcholine chloride (Sigma), phentolamine mesylate (Ciba-Geigy), PV extract, fractions I, II, and III, harpagide, and 8-*O*-acetylharpagide (obtained in our laboratory).

RESULTS AND DISCUSSION

The MeOH residue (MR), dissolved in EtOH 95°, exhibited a contracting action on the guinea pig ileum, while the H_2O-soluble part (PV), fractions II and III, were without effect. Fraction I was in some experiments contracting and in oth-

ers relaxant, but not dose-dependent and was consequently discarded.

In the guinea pig colon, a dose-dependent relaxant activity of PV in a dose range of $3 \cdot 10^{-4}$–$1 \cdot 10^{-3}$ g/ml was demonstrated.

Fractions II and III, chemically identical, also exhibited the same relaxant effect in an apparent dose range of $1 \cdot 10^{-5}$–$1 \cdot 10^{-3}$ g/ml, comparable to that of noradrenaline. The dose-effect curve was shifted to the left with respect to that obtained with PV (Figure 1).

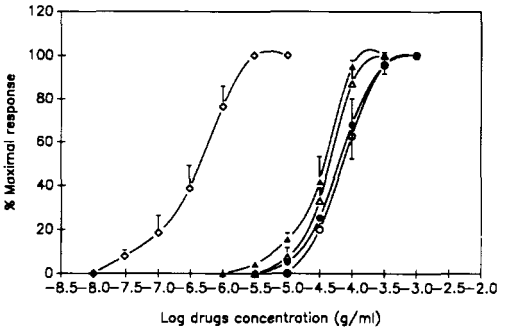

FIGURE 1. Agonistic activity (relaxation) of PV (○), fraction II (●), iridoid mixture (△), 8-acetylharpagide (▲), and noradrenaline (◇) on isolated guinea-pig colon. pD_2 values of noradrenaline and 8-*O*-acetylharpagide are 5.70±0.15 and 4.18±0.19, respectively. Each value represents the mean ± SE of 3–5 experiments.

The isolated iridoids, in a mixture of 8-*O*-acetylharpagide and harpagide in a ratio of about 3:2, maintained the above activity with a further shift to the left of the dose-response curve (Figure 1). Of the two isolated iridoids only one (8-*O*-acetylharpagide) demonstrated a greater degree of activity with respect to the mixture. In all the tests the observed activity was antagonized by phentolamine $1 \cdot 10^{-5}$ g/ml.

The MeOH residue, dissolved in 95% EtOH, was inactive on guinea pig aortic spiral; PV and fractions II and III elicited on this smooth musculature a dose-dependent contraction. They exhibited an intrinsic activity comparable to that of noradrenaline. The dose-effect curve was shifted to the left by the concentrated fractions II and III, and an increase in the activity was also detectable when the mixture of iridoids or 8-*O*-acetylharpagide was examined (Figure 2). The contracting activity was antagonized by phentolamine $1 \cdot 10^{-5}$ g/ml.

The lack of any activity of the MeOH

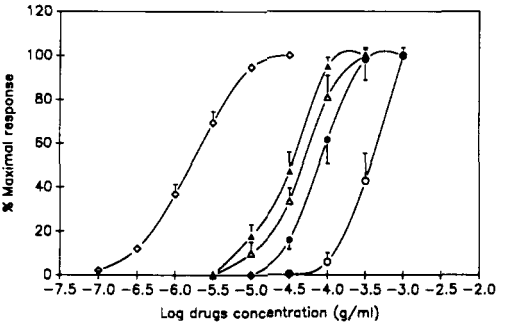

FIGURE 2. Agonistic activity (contraction) of PV (○), fraction II (●), iridoid mixture (△), 8-*O*-acetylharpagide (▲), and noradrenaline (◇) on isolated guinea pig aorta. pD_2 values of noradrenaline and 8-*O*-acetylharpagide are 5.42±0.09 and 4.24±0.11 respectively. Each value represents the mean ± SE of 3–5 experiments.

residue (MR) on aortic preparation led us to reject the first hypothesis of a tryptaminergic or myotropic involvement in the action mechanism of the antihemorrhagic effect of *A. reptans*, as could be suggested from the observations on ileum segments.

The appearance of a contracting activity on vascular smooth musculature for PV and fractions II and III can be ascribed to an activation of α-adrenoceptors. The hypothesis is supported by the results obtained on colic smooth musculature, where activation of both α- and β-adrenoceptors produces an inhibitory tone on spontaneous rhythmic activity, and by antagonism exerted by phentolamine.

The resting tone of ileal musculature, in our experimental condition, permits the recording of contractions but not of relaxations, and consequently it is

not surprising the inefficiency of these fractions in the preparation.

The maintaining of the above effect on aortic spiral by fractions II and III, with a shift to the left of the dose-response curve with respect to that of PV, is evidence that the active principle is being concentrated. The isolation of two iridoids, only one (8-*O*-acetylharpagide) with activity, further increased the shift to the left of the dose-effect curve.

It can be postulated that the antihemorrhagic effect of *A. reptans* is due to a vasoconstriction mediated by involvement of vasal α-adrenoceptors. The high concentrations of the various fractions or of the active principle (iridoid) required in vitro to obtain the pharmacological effects would not be a limiting factor for the therapeutic effect because the usual topical applications of the *Ajuga* plant parts permits a therapeutically efficacious concentration.

We can conclude that the iridoid 8-*O*-acetylharpagide, already isolated from *A. reptans* (15,16) and from other species of *Ajuga* and other families (24–25), appears to be the active principle, or one of the possible active principles. Since the applied chemical screening methods did not permit the histochemical localization of the above iridoids, there is no experimental evidence of the site of synthesis and/or storage of these compounds.

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