SUPPLEMENTARY MATERIALS

***Harpagophytum procumbens* extract potentiates morphine antinociception in neuropathic rats**

Carmela Parenti^a, Giuseppina Aricò^{b*}, Marzio Pennisi^c, Alessandro Venditti^{d,e}, Giovanna M. Scoto^a.

^{a)} Department of Drug Sciences, Pharmacology and Toxicology section,

^{b)} Department of Drug Sciences, Medicinal Chemistry section,

^{c)} Department of Mathematics and Computer Science, University of Catania, Viale A. Doria 6, 95125 Catania, Italy.

^{d)} Department of Chemistry, "Sapienza" University of Rome, P.le A. Moro 5, 00185 Rome, Italy,

^{e)} Department of Environmental Biology, "Sapienza" University of Rome, P.le A. Moro 5, 00185 Rome, Italy

*Corresponding author: Giuseppina Aricò

Email: garico@unict.it

Telephone number 00390957384025

Fax number 00390957384214

Abstract

The association of opioids and non steroidal anti-inflammatory drugs, to enhance pain relief and reduce the development of side effects, has been demonstrated. Given many reports concerning the antinociceptive and anti-inflammatory effects of *Harpagophytum procumbens* extracts, the aim of our study was to investigate the advantage of a co-administration of a subanalgesic dose of morphine preceded by a low dose of *Harpagophytum procumbens* to verify this therapeutically useful association in a neuropathic pain model.

Time course, registered with the association of the natural extract, at a dose that does not induce an antinociceptive effect, followed by a subanalgesic dose of morphine showed a well-defined antiallodynic and antihyperalgesic effect, suggesting a synergism as a result of the two-drug association. *Harpagophytum procumbens* cooperates synergistically with morphine in resolving hyperalgesia and allodynia, two typical symptoms of neuropathic pain. The results support the strategy of using an adjuvant drug to improve opioid analgesic efficacy.

Keywords: hyperalgesia, allodynia, synergism, opioid, persistent pain, *Harpagophytum procumbens*.

3. Experimental

3.1. Drugs

H. procumbens standardized dried extract was kindly donated by Aboca (San Sepolcro, Italy), batch number 10J1398. Morphine hydrochloride was purchased from S.A.L.A.R.S. (Como, Italy) and Zoletil® from Farmavet (Catania Italy). All drugs were dissolved in 0.9% sterile saline.

3.2. Plant Extract and harpagoside determination method

The dried root from Namibia (Africa), was extracted with ethanol:water 70:30 (v:v). At the end of extraction, ethanol was evaporated under vacuum and the resulting mixture was freeze-dried yielding the correspondent extract (30% of the starting weight). The samples of *H. procumbens* dried extract were standardized at 2% of harpagoside content.

Harpagoside standard (purity 99.9%, HPLC grade) was purchased from Phytolab. Methanol HPLC grade was purchased from Merck (Darmstadt, Germany). Syringe filters were purchased from GE Osmonic Labstore (Minnetonka, MN). Water was purified using a Modupure Plus model LBMPP 20 1200 (Continental Water Systems Corporation, United States).

HPLC analysis were performed using an Agilent Technologies (Palo Alto, CA) modular 1100 system consisting of a vacuum degasser, a binary pump, a thermostated autosampler, a thermostated column compartment, and an UV VIS DAD detector. A Prodigy RP-C8 (4.6 mm × 250 mm, 100 Å, 5mm, Phenomenex, United States) analytical column protected by a RP-C8 security guard cartridge (4 mm × 3 mm) was used. The mobile phase was composed of water and Methanol and pumped at 1 mL/min in 25 min. The elution ramp is: at 0 min 50/50 water methanol, at 15 min 30/70 water/methanol and then until 20 min 10/90 water/methanol. Stop time at 25 min the autosampler and the column compartment were thermostated at 20 °C. 0.25 g of *H. procumbens* standardized dried extract sample was treated with 50 mL of the mobile phase (Methanol/Water 80/20) for 30min, at room temperature under ultrasonic treatment. The resulting mixture was centrifuged, filtered into a volumetric flask, and brought to the final volume of 50 mL with the same solvent mixture. All of the extracts were filtered through 0.45 mm Cellulose acetate syringe filter before HPLC analysis. The stock solution was diluted to obtain samples with harpagoside concentration ranging from 0.025 to 0.1 mg/mL. Three 20 µL injections of each concentration were made. The calibration curve was obtained by plotting the mean peak areas vs the corresponding concentrations.

3.3. Animals

Experiments were conducted on male Sprague–Dawley rats (Harlan, San Pietro al Natisone (UD), Italy), weighing 180–200 g. The animals were kept at a constant room temperature ($25 \pm 1^\circ\text{C}$) under a 12:12 h light and dark cycle with free access to food and water. Each rat was used for only one experiment. All tests were performed at room temperature (22–24 °C) between 08:00 and 15:00. Experimental procedures were approved by the local ethical committee and the Institutional Animal Care and Use Committee (IACUC), and all experiments were conducted in accordance with International Guidelines as well as European Communities Council Directive and National Regulations (EEC Council 86/609 and DL 116/92).

3.4. Experimental procedures

Animals were randomly assigned to one of groups with 8–10 animals per group. Mechanical allodynia and thermal hyperalgesia thresholds were assessed in different animal groups, as follows:

Groups 1 and 2: assessment of time course of allodynia and hyperalgesia in vehicle treated CCI rats;

Groups 3-8: i.p. administration of *H. procumbens*. (400, 600, 800 mg/kg) to CCI rats

Groups 9-14: i.p. administration of morphine. (3, 4, 5 mg/kg) to CCI rats

Groups 15-16: *H. procumbens* (400 mg/kg i.p.) plus morphine (3 mg/kg i.p.).

After the completion of the experiment, the animals were sacrificed under deep anesthesia.

3.5. Chronic constriction injury model of neuropathic pain

CCI was produced according to Bennett and Xie (Bennett & Xie 1998; Parenti et al. 2013). Briefly, animals were anaesthetized with Zoletil® 50 mg/kg, and an incision was made just below the hip bone, parallel to the left common sciatic nerve. The sciatic nerve was exposed and four ligatures (4/0 chromic silk, Ethicon) were loosely tied around the nerve at the level of the mid thigh and proximal to the trifurcation of the nerve at about 1 mm spacing, until a brief twitch in the respective hind limb was observed.

3.6. Behavioral testing

3.6.1. Mechanical allodynia

The assessment of tactile allodynia consisted of measuring the withdrawal threshold of the hind paw in response to probing with a series of calibrated von Frey's filaments. The rat was placed in a clear plastic testing chamber with a wire mesh bottom and allowed to acclimatize for 20 min. The ventral surface of the hind paw was mechanically stimulated from below with an ascending series of graded von Frey's filaments with bending forces ranging from 0.02 to 30 g. The withdrawal threshold was determined by the "up-down" method of sequentially increasing and decreasing the stimulus strength (Dixon 1980; Scoto et al. 2009) and was expressed as the mean withdrawal threshold.

3.6.2. Thermal hyperalgesia

Thermal hyperalgesia was quantified using the method described by Hargreaves et al. (Hargreaves et al, 1988). Briefly, rats were placed in a plexiglass box (17 x 23 x 14 cm) on a glass surface of the apparatus (Plantar test, Ugo Basile, Italy), and a beam of radiant heat was applied through the glass to the plantar surface of the left hind paw. Rats were allowed to habituate to the apparatus until exploratory behavior was no longer observed. The basal pre-drug latency was established between 8 and 10 s and was calculated as the average of two measurements performed at 5 min intervals with a cut-off latency of 15 s to avoid tissue damage (Parenti et al. 2013).

3.7. Statistical analysis

Data are expressed as the mean $\pm$ SEM. Intergroup comparisons were assessed by one- or two-way analysis of variance (ANOVA) when appropriate, followed by Post-hoc tests (Dunnett's test for one-way or Bonferroni for two-way ANOVA). A *P* value less than 0.05 ($P < 0.05$) was considered statistically significant.

References.

- Bennett GJ, Xie YK. 1998. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain*. 82(1),87–107.
- Dixon WJ. 1980. Efficient analysis of experimental observations. *Annual Review of Pharmacology and Toxicology*. 20:441–462.
- Hargreaves K, Dubner R, Brown F, Flores C, Joris J, 1988. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain*. 32(1):77–88.
- Parenti C, Turnaturi R, Arico G, Gramowski-Voss A, Schroeder OH-U, Marrazzo A, Prezzavento, O, Ronsisvalle S, Scoto GM, Ronsisvalle G, Pasquinucci L. 2013. The multitarget opioid ligand LP1's effects in persistent pain and in primary cell neuronal cultures. *Neuropharmacology*. 71:70-82.
- Scoto GM, Arico G, Iemolo A, Ronsisvalle S, Parenti C. 2009. Involvement of the Nociceptin/Orphanin FQ-NOP receptor system in the ventrolateral periaqueductal gray following mechanical allodynia in chronic pain. *Life Sciences*. 85(5-6):206-210.