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Attenuation of capsaicin-induced acute and visceral nociceptive pain by α- and β-amyrin, a triterpene mixture isolated from *Protium heptaphyllum* resin in mice

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Abstract

The triterpene mixture, α- and β-amyrin, isolated from *Protium heptaphyllum* resin was evaluated on capsaicin-evoked nociception in mice. Orally administered α- and β-amyrin (3 to 100 mg/kg) significantly suppressed the nociceptive behaviors—evoked by either subplantar (1.6 μg) or intracolonic (149 μg) application of capsaicin. The antinociception produced by α- and β-amyrin against subplantar capsaicin-induced paw-licking behavior was neither potentiated nor attenuated by ruthenium red (1.5 mg/kg, s.c.), a non-specific antagonist of vanilloid receptor (TRPV1), but was greatly abolished in animals pretreated with naloxone (2 mg/kg, s.c.), suggesting an opioid mechanism. However, participation of α_2-adrenoceptor involvement was unlikely since yohimbine (2 mg/kg, i.p.) pretreatment failed to block the antinociceptive effect of α- and β-amyrin in the experimental model of visceral nociception evoked by intracolonic capsaicin. The triterpene mixture (3 to 30 mg/kg, p.o.) neither altered significantly the pentobarbital sleeping time, nor impaired the ambulation or motor coordination in open-field and rota-rod tests, respectively, indicating the absence of sedative or motor abnormality that could account for its antinociception. Nevertheless, α- and β-amyrin could significantly block the capsaicin (10 mg/kg, s.c.)-induced

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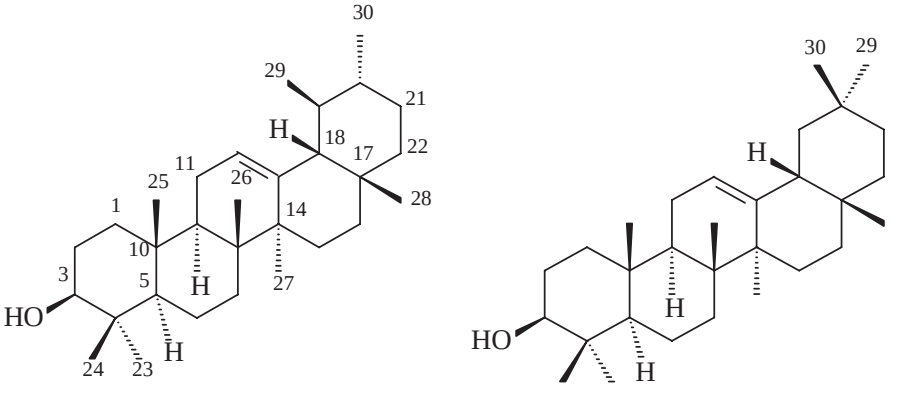
hyperthermic response but not the initial hypothermia. These results suggest that the triterpene mixture, α- and β-amyrin has an analgesia inducing effect, possibly involving vanilloid receptor (TRPV1) and an opioid mechanism.

Keywords: *Protium heptaphyllum*; Triterpene mixture; α- and β-amyrin; Capsaicin; Antinociception; Mouse

Introduction

Many plant-derived substances are attractive sources for developing new analgesic agents. Several triterpenoid compounds have been shown to produce promising results in experimental and clinical studies (Calixto et al., 2000; Almeida et al., 2001; Takayama et al., 2002). *Protium heptaphyllum* March (Burseraceae), popularly known as almécega, is a medicinal plant that grows abundantly in Amazon region and in various other parts of Brazil. The resinous exudate collected from the trunk wood of this plant in its natural form is a reputed folk remedy with anti-inflammatory, analgesic, expectorant and wound healing actions (Pernet, 1972; Correia, 1984; Siani et al., 1999). Phytochemical studies on resin revealed the presence of several monoterpenes and some pentacyclic triterpenes that include a mixture of α- and β-amyrin (Susunaga et al., 2001; Bandeira et al., 2002). In general, pentacyclic triterpenes manifest anti-inflammatory, antiulcer, antinociceptive and antitumoral properties (Finney and Tarknoy, 1960; Inoue et al., 1990; Otuki et al., 2001). Our previous studies established in mice, the gastroprotective function of crude resin and its major terpenoid component, α- and β-amyrin (Fig. 1) against ethanol-induced damage, which possibly involves the activation of capsaicin-sensitive primary afferents (Oliveira et al., 2004a,b).

Capsaicin, the pungent ingredient of red peppers applied topically or injected into the skin of humans or experimental animals, is known to stimulate the vanilloid receptor (Transient Receptor Potential cation channel V1 or TRPV1) located on polymodal C-fibers, but also in other tissues and initiates a complex cascade of events, including neuronal excitation and release of pro-inflammatory mediators,



α-Amyrin β-amyrin

Fig. 1. Chemical structures of α-amyrin and β-amyrin.

desensitization of receptor, and neuronal toxicity (Szolcsanyi, 1977; Caterina et al., 1997). Excitation of sensory neurons by capsaicin induces a burning pain followed by a refractory state that produces analgesic effects against a subsequent nociceptive stimulus (Szallasi and Blumberg, 1999). The functional role of TRPV1 in nociception has been established by the use of TRPV1 antagonists, and TRPV1 knockout mice (Caterina et al., 2000; Walker et al., 2003). The desensitization induced after TRPV1 agonists points to the utility of these agents in the suppression of neuropathic pain, whereas TRPV1 antagonists as valuable agents for the treatment of inflammatory hyperalgesia and pain (Lopez-Rodriguez et al., 2003). It was therefore of interest in the present study to examine the possible modulation of capsaicin-induced acute pain and visceral nociception by α- and β-amyrin, the triterpene mixture isolated from *Protium heptaphyllum* resin.

Materials and methods

Animals

Male Swiss mice (20–25 g) were used. Experimental groups consisted 8 to 10 animals per group. They were housed at 24° ± 2 °C under a 12-h light/12-h dark cycle and had free access to standard pellet diet (Purina chow) and tap water. The animals were deprived of food for about 15 h before experimentation, but had free access to drinking water. Animal Care and Use Committee of our Institute approved the experimental protocols in accordance with the ethical guidelines for the investigation of experimental pain in conscious animals (Zimmermann, 1983).

Chemicals

The following chemicals were used: capsaicin (Calbiochem, San Diego, California, USA), naloxone (Dupont, Garden City, USA), morphine hydrochloride (Cristalia, São Paulo, Brazil), pentobarbitone sodium (Abbotts Labs), Ruthenium red, yohimbine and clonidine hydrochloride (Sigma, MO, USA). Capsaicin was dissolved in a solution containing 10% ethanol, 10% Tween 80, and 80% saline. Morphine, yohimbine, clonidine, pentobarbitone, and naloxone were dissolved in 0.9% NaCl solution. Ruthenium red was diluted in 0.9% NaCl solution.

The triterpene, α- and β-amyrin, was isolated as a mixture (63:37) from the crude resin collected from trunk wood of *Protium heptaphyllum* (Fig. 1) according to a previously described method (Oliveira et al., 2004b). The triterpenoid was dissolved in 0.8% NaCl solution, using 3% Tween 80.

Effect of α- and β-amyrin mixture and morphine on acute nociceptive behavior-evoked by subplantar injection of capsaicin

Mice were habituated for the test environment for 30 min before the test in standard transparent cages (22 × 15 × 12.5 cm). After this period, 20 μl of capsaicin (1.6 μg,) or vehicle, was injected intradermally into the heel pad using a microsyringe with a 26-gauze needle, and personnel unaware of the treatments recorded the amount of time the animals spent licking the injected paw for a period of 5 min (Santos and Calixto, 1997). The test doses of α- and β-amyrin (3, 10, 30, and 100 mg/kg, p.o.), morphine (5 mg/kg, s.c.) or the vehicle used for dissolving α- and β-amyrin (10 ml/kg, p.o.) were administered 60 min before

capsaicin. A separate control group that received subplantar injection of only vehicle (capsaicin) was included in the study. To verify the possible role of endogenous opioids in the antinociceptive effect of α- and β-amyrin, naloxone (2 mg/kg, i.p.)-pretreated groups of animals were also included, in which case naloxone was administered 15 min before morphine or triterpenoid mixture. The dose selection of capsaicin (1.6 μg) to induce nociceptive behavior in mice was based on our pilot experimentation and literature citation.

In a separate experiment, a possible interaction between α- and β-amyrin and ruthenium red (a non-selective capsaicin antagonist) was studied. Four groups of mice (eight animals in each) were included in the study and were respectively treated with the vehicle alone, α- and β-amyrin alone (10 mg/kg, p.o.), ruthenium red alone (1.5 mg/kg, s.c. on 3 successive days), or the combination of ruthenium red and α- and β-amyrin. One hour following the drug treatments, the paw-licking response to subplantar injections of capsaicin (1.6 μg in 20 μl) was noted in each group of animals.

Effect of α- and β-amyrin mixture and morphine on nociceptive behaviors induced by intracolonic administration of capsaicin (visceral nociception)

Visceral nociceptive test was performed as described previously (Laird et al., 2001; Kawao et al., 2004). Briefly, mice ($n=8$ to 10 per group) were acclimated to the experimental environment for 30 min. Using a cannula with a rounded tip (1.6 mm), 50 μl of capsaicin (149 μg) was administered to each mouse into the colon at 4 cm from the anus, after application of vaseline to the perianal region to avoid the stimulation of somatic areas by contact with capsaicin. Immediately after capsaicin administration, the number of visceral pain-related nociceptive behaviors, like abdominal licking, stretching, squashing of the lower abdomen against the floor, and abdominal retractions, was observed blind for a 30-min period (Laird et al., 2001). The test doses of α- and β-amyrin (3–100 mg/kg, p.o.), clonidine (0.1 mg/kg, p.o.), yohimbine (2 mg/kg, s.c.), and their combinations or the vehicle used for dissolving α- and β-amyrin (10 ml/kg, p.o.) were administered 60 min before the intracolonic administration of capsaicin. A separate control group that received intraplantar injection of only vehicle (capsaicin) was also included in the study. Based on preliminary experiments, a dose of 149 μg of capsaicin was selected for intracolonic administration, and at this dose, mice demonstrated more consistent and reproducible nocifensive behaviors.

Effect of α- and β-amyrin mixture on thermic responses induced by capsaicin

Groups of male mice ($n=8$) were treated with the vehicle of α- and β-amyrin (10 ml/kg, p.o.), α- and β-amyrin mixture (10 and 100 mg/kg, p.o.), or ruthenium red (1.5 mg/kg, s.c., twice daily for 3 days) 1 h before the injection of capsaicin (10 mg/kg, s.c.). Rectal temperature of animals was measured before (0 h) and 30, 60, 90, 120, and 150 min and at 24 h and 48 h post-injection of capsaicin, with a digital clinical thermometer (Becton Dickinson, Brazil) inserted 2 cm into the rectum.

Effect of α- and β-amyrin mixture on sleeping time induced by pentobarbital sodium

The test was carried out in male mice ($n=8$ to 10 per group) treated as follows: group 1 received the vehicle of α- and β-amyrin (10 ml/kg, p.o.); groups 2, 3 and 4 received the α- and β-amyrin mixture at doses of 3, 10 and 30 mg/kg, p.o., respectively. One hour after treatments, all animals groups were given

pentobarbital sodium (50 mg/kg, i.p.) and the duration of sleep (min) in each animal was observed. Loss of righting reflex to recovery was recorded as the sleeping time (Darias et al., 1998).

Effect of α- and β-amyrin mixture on locomotor activity in open-field test

The open-field area was made of acrylic (transparent walls and black floor. 30 × 30 × 15 cm) divided into nine squares of equal area. Four groups of animals ($n = 8–10$ per group) were treated as above with the vehicle or the mixture of α- and β-amyrin in three doses. The number of squares crossed with the four paws was noted for each animal, during a 4-min period (Archer, 1973).

Effect of α- and β-amyrin mixture on motor coordination in rota-rod test

The test was carried out according to the method described earlier (Rosland et al., 1990). The apparatus consisted of a horizontal bar with a diameter of 5 cm, subdivided into four compartments (INSIGHT, RT-2002, Brazil). The mice were placed on the bar rotating at a speed of 4 rpm and mice that were able to remain on the rod longer than 120 s were selected 24 h before the test. They were divided into four groups ($n = 8–10$ per group) and were treated as above with the vehicle or the mixture of α- and β-amyrin in three doses. One hour later, for each animal, the time of permanence (s) on the bar during a 2-min period was registered.

Statistical analysis

The results are presented as the mean ± S.E.M. of 8 to 10 animals per group. Statistical comparison of the data was performed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test

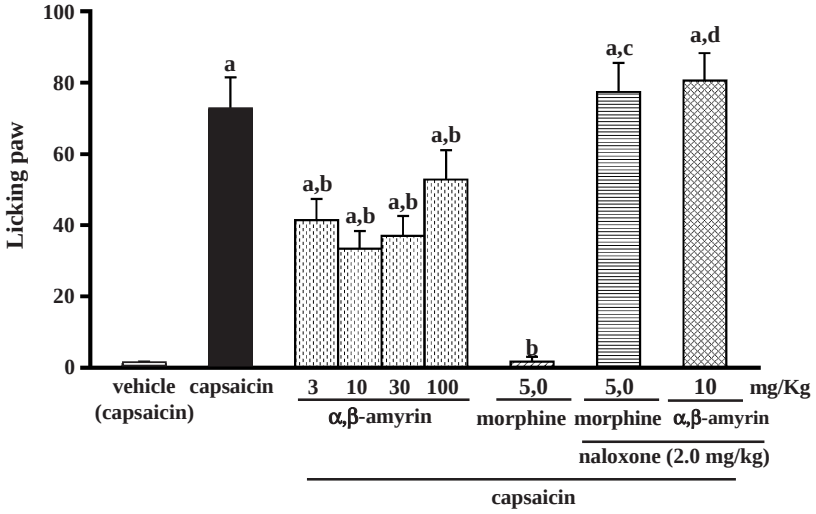


Fig. 2. Effects of triterpenoid mixture, α,β-amyrin and morphine on intraplantar capsaicin (1.6 μg)-induced acute nociception in mice. Data are reported as mean ± S.E.M from 8 to 10 animals; [a]*$P < 0.05$ vs. vehicle control (capsaicin); [b]*$P < 0.05$ vs. capsaicin; [c]*$P < 0.05$ vs. morphine; [d]*$P < 0.05$ vs. α- and β-amyrin (10 mg/kg, p.o.).

Table 1
Effects of α- and β-amyrin mixture and ruthenium red treatments on paw-licking response to subplantar capsaicin in mice

Treatment	Dose (mg/kg)	Paw licking (s)
Control (vehicle)	–	60.50 ± 5.97
Ruthenium red (s.c., twice daily for 3 days)	1.5	36.87 ± 5.538*
α,β-Amyrin (p.o.)	10	37.77 ± 4.87*
Ruthenium red (s.c.)+α,β-amyrins (p.o.)	1.5+10	35.0 ± 3.12*

Each group represents mean ± S.E.M. for 8–10 animals. Capsaicin (1.6 μg in 20 μl) was injected 1 h after completion of pretreatments with vehicle, α,β-amyrin or ruthenium red.
* $P<0.01$ vs. control (ANOVA followed by Student t-test).

for multiple comparisons. P-values less than 0.05 ($P<0.05$) were considered as indicative of statistical significance.

Results

Fig. 2 shows the suppressive effect of triterpene mixture on paw-licking response to subplantar injection of capsaicin (1.6 μg). The subplantar injection of vehicle used for capsaicin produced only a negligible nociceptive response. When compared to capsaicin control, the extent of inhibitions of paw-licking response for the tested doses of 3, 10, 30 and 100 mg/kg (p.o.) α- and β-amyrin, and of morphine (5 mg/kg, s.c.) were in the order of 43%, 54%, 49%, 27%, and 97%, respectively. At 30 and 100 mg/kg, there was a diminished trend in the attenuation of nociception. Naloxone (2 mg/kg, i.p.) pretreatment completely reversed the antinociception produced by 5 mg/kg (s.c.) of morphine as well as of 10 mg/kg (p.o.) α- and β-

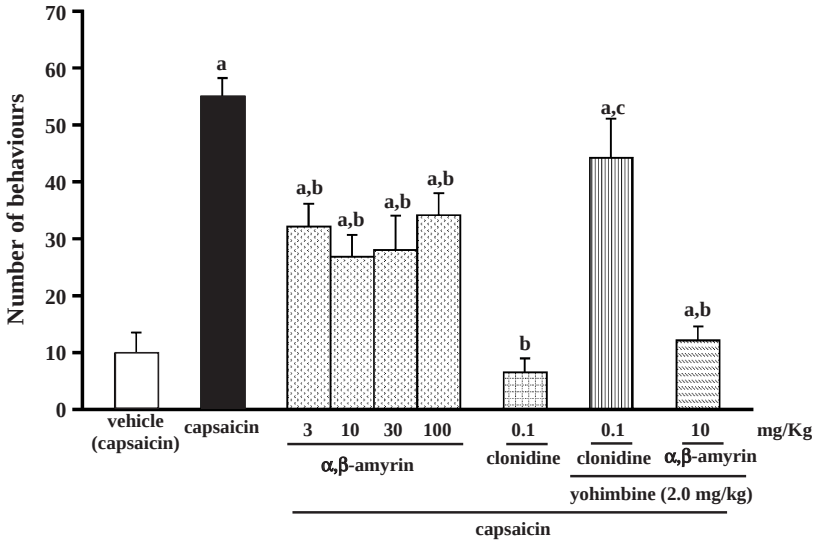


Fig. 3. Effects of triterpenoid mixture, α,β-amyrin and clonidine on nociception induced by intracolonic capsaicin (149 μg) in mice. Data are reported as mean ± S.E.M from 8 to 10 animals; [a]*$P<0.05$ vs. vehicle control (capsaicin); [b]*$P<0.05$ vs. capsaicin; [c]*$P<0.05$ vs. clonidine; [d]*$P<0.05$ vs. α- and β-amyrin (10 mg/kg, p.o.).

amyrin, a dose that caused maximal suppression of paw licking. The results obtained in the experiment designed to verify a possible interaction between ruthenium red (1.5 mg/kg/day, s.c. for 3 days) and α- and α-amyrin (10 mg/kg) on capsaicin-induced nociception are shown in Table 1. Like α- and β-amyrin, ruthenium red produced a similar potency in the inhibition of nociception. When these two substances were co-administered to mice, there was no additive or antagonistic effect on capsaicin-induced nociception.

Fig. 3 summarizes the number of visceral nociceptive behaviors evoked by intracolonic application of capsaicin (149 μg) in vehicle, α- and β-amyrin, and morphine-pretreated mice. Intracolonic administration of capsaicin to vehicle (α- and β-amyrin)-treated group of mice manifested significantly increased (5.6-fold) number of nociceptive behaviors than to solvent (capsaicin). Pretreatment with α- and β-amyrin (3, 10, 30, and 100 m/kg, p.o.) and clonidine (0.1 mg/kg, p.o.) resulted in significantly reduced number of nociceptive behaviors and the extent of reductions were in the order of 42%, 56%, 49%, 38%, and 88%, respectively. At 30 and 100 mg/kg, there was a diminished trend in the attenuation of nociception. Yohimbine (2 mg/kg, i.p.) pretreatment significantly reversed the antinociception produced by clonidine but not of α- and β-amyrin (10 mg/kg, p.o.), a dose that caused maximal suppression of nociceptive behaviors.

Fig. 4 depicts the capsaicin-evoked changes in rectal temperature of mice and the pretreatment effect of α- and β-amyrin and ruthenium. Capsaicin given subcutaneously in a dose of 10 mg/kg induced an initial hypothermia (during the 30- to 150-min period of observation), followed by a hyperthermia (24 and 48 h). At the tested doses, both α- and β-amyrin (10 mg/kg, p.o.) and ruthenium red (1.5 mg/kg/day, s.c., for 3 days) failed to modify the hypothermic response of capsaicin. However, the hyperthermic response seen at 24 and 48 h after capsaicin was not apparent in ruthenium red-pretreated animals. In contrast, mice pretreated with α- and β-amyrin at 10 mg/kg but not with 100 mg/kg manifested absence of hyperthermia.

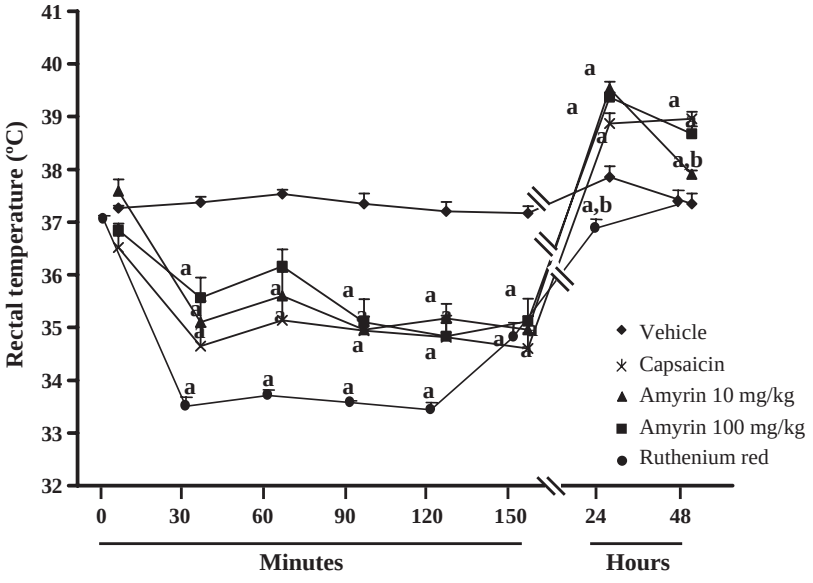


Fig. 4. Effects of triterpenoid mixture, α- and β-amyrin and ruthenium red, a non-selective TRPV1 antagonist on thermic responses induced by capsaicin (10 mg/kg, s.c.) in mice. Capsaicin was injected 1 h after completion of pretreatments with vehicle, α- and β-amyrin (10 mg/kg, p.o.), or ruthenium red (1.5 mg/kg, s.c., twice a day for 3 days). Data are reported as mean ± S.E.M from 8 to 10 animals [a]*$P<0.05$ vs. vehicle control (capsaicin); [b]*$P<0.05$ vs. capsaicin.

Table 2
Effect of treatment with α- and β-amyrin mixture on pentobarbitone (50 mg/kg, i.p.) sleeping time, and in open-field and rota-rod tests with mice

Treatment	Dose (mg/kg, p.o)	Sleeping time (min)	Open field (number of crossing)	Rota-rod performance (s)
Vehicle	–	56.57 ± 3.14	41.11 ± 2.43	117.37 ± 2.62
α,β-Amyrin	3	43.42 ± 3.34	45.30 ± 3.26	120.00 ± 0.00
	10	44.5 ± 4.63	41.88 ± 3.71	114.14 ± 3.82
	30	61.75 ± 4.21	36.62 ± 2.58	119.28 ± 0.71

Each group represents mean ± S.E.M. for 8–10 animals.

Table 2 shows the effects of orally administered α- and β-amyrin mixture on pentobarbitone (50 mg/kg, i.p.)-induced sleeping time, behavior in open-field and on rota-rod performance in mice. At the doses tested (3, 10, and 30 mg/kg), α- and β-amyrin mixture showed no significant influence on pentobarbitone sleeping time, the number of crossing in open-field test or on the rota-rod performance.

Discussion

In the present study, we observed that the capsaicin-evoked nociceptive pain behaviors were significantly attenuated in mice pretreated with α- and β-amyrin, a natural pentacyclic triterpenoid isolated from the resin of *P. heptaphyllum*. It has been well established that capsaicin induces nociceptive pain-related behaviors in rodents following subplantar (paw licking), or intracolonic (abdominal licking, stretching, squashing of the lower abdomen against the floor, and abdominal retractions) application, and several reports reveal that naturally occurring compounds, like terpenoids, unsaturated dialdehydes and phenolic ketones can suppress these behaviors (Szallasi and Blumberg, 1999; Laird et al., 2001; Otuki et al., 2001; Dedov et al., 2002; Luo et al., 2003; Andre et al., 2004; Kawao et al., 2004).

The α- and β-amyrin mixture could effectively suppress the pain-related nociceptive behaviors evoked by subplantar or intracolonic capsaicin almost to a similar magnitude (Figs. 2 and 3). At smaller doses, it showed a greater efficacy whereas at higher doses (30 and 100 mg/kg), evidenced a diminished tendency, resulting in U-shaped dose–response curve. It implies that amyrin mixture may have a dual action on capsaicin response. In our earlier studies (Oliveira et al., 2004b), we observed a similar tendency in its gastroprotective effect against ethanol-induced gastric injury in mice. The mechanism underlying the antinociceptive action of triterpenoid seems to be related to activation of the opioid system as naloxone, an opioid receptor antagonist reversed its antinociceptive effect in subplantar capsaicin pain model.

The capsaicin (vanilloid) receptor TRPV1 is a non-selective cation channel and is activated not only by capsaicin but also by noxious heat, protons (low pH) and direct phosphorylation by protein kinase C (PKC) (Correll et al., 2004). Capsaicin excites a significant fraction of nociceptors and promotes behavioral responses like paw licking and hypothermia, which are thought to be inhibited by capsaicin antagonists such as capsazepine and ruthenium red (Perkins and Campbell, 1992; Szallasi and Blumberg, 1999). Mice lacking TRPV1 receptor (knockout mice) show a marked reduction in nocifensive behavioral responses (Caterina et al., 2000). In the present study, to gain evidence on the possible mechanism through which, α- and β-amyrin exerts antinociception, experiments were realized using ruthenium red, a non-selective capsaicin antagonist in the model of nociception induced by subplantar injection of capsaicin. The results obtained show a similar extent of inhibition by both ruthenium red and

α- and β- amyrin. Further, when these two substances were co-administered to mice, there was no additive or antagonistic effect on capsaicin-induced nociception, indicating that there is no possible interaction of α- and β-amyrin with TRPV1. These results suggest that both ruthenium red and α- and β-amyrin can produce antinociceptive effect, which may be mediated by inhibiting the membrane cation channel activated by capsaicin.

Next, we examined whether α- and β-amyrin and ruthenium red can antagonize the hypothermic response induced by subcutaneously administered capsaicin in mice. Consistent with earlier observations (Szikszay et al., 1982), capsaicin at the dose of 10 mg/kg produced pronounced hypothermia followed by a sustained hyperthermia, which persisted at least for 48 h. Neither ruthenium red nor the α- and β-amyrin was able to block the hypothermic response of capsaicin. However, ruthenium red could completely abolish the hyperthermic response of capsaicin whereas with α- and β-amyrin, suppression of hyperthermia was noticed only at 48 h. Failure to block the hypothermic effect of capsaicin by α- and β-amyrin and ruthenium red may imply that these agents lack specificity to act as TRPV1 antagonists in mice. In support of this view were the molecular pharmacological studies of Correll et al. (2004) that question the selectivity of even capsazepine as a TRPV1 antagonist and suggest that it may be an ineffective TRPV1 antagonist for in vivo models of inflammatory pain in mouse.

The α_2-adrenergic mechanisms modulate colorectal sensations and clonidine, an α_2-adrenoceptor agonist induces antinociception (Pertovaara and Kalmari, 2003). In this study, we determined a likely role of α_2-adrenoceptors in the visceral antinociceptive effect of α- and β-amyrin, using clonidine as a reference compound. Pretreatment with the α_2-adrenoceptor agonist, yohimbine significantly blocked the antinociceptive effect of clonidine, but not that of α- and β-amyrin, suggesting the absence of sympathetic component in its effect.

Constant activation and sensitization of polymodal C-type primary afferents are an important component of inflammatory pain. These fibers release neuropeptides, such as substance P and calcitonin-gene related peptide (CGRP), which contribute to neurogenic inflammation in the periphery and might cause increased spinal excitability (wind up) and central sensitization responsible for hyperalgesia and allodynia (Woolf, 1992). Visceral pain is an important symptom of many clinical conditions, such as irritable bowel syndrome wherein sensory afferents in the muscle wall and mucosa are activated by distention during peristalsis and or sensitized by a variety of chemical mediators generated from both within and outside the gut wall to cause central sensitization conveying sensory information from the gut to the central nervous system (Grundy, 2004). Once central sensitization is established, larger doses of analgesics are needed and, therefore, treatment before pain progresses may arrest the central sensitization and reduce the requirement of analgesic (Woolf and Chong, 1993). Capsaicin application is considered a suitable model to study peripheral/central sensitization following the activation of nociceptors in primary afferents. In the tested models of capsaicin-induced pain, the triterpenoid mixture, α- and β-amyrin, manifested a suppressive effect on nociceptive behaviors, and therefore, this compound may have a therapeutic potential for treating neuropathic and or inflammatory pain. However, studies of the effects of α- and β-amyrin in experimental models of thermal hyperalgesia and neuropathic pain are warranted.

Drugs that impair motor activity or induce sedation may give false-positive/negative results in nociceptive tests. We therefore sought to verify such effects of triterpene mixture on pentobarbitone sleeping time, which detects sedation (Williamson et al., 1996); rota-rod performance, a good index for neurological deficits including sedation, muscle relaxant and impairment of motor activity; and in open-field test that detects motor incoordination (Novas et al., 1988; Pu et al., 1995). The triterpene mixture, in

the dose range of 3 to 30 mg/kg (p.o.), failed to alter significantly the pentobarbitone-sleeping time, the ambulation in open-field test or the rota-rod performance. These results indicate that α- and β-amyrin exerts analgesia without causing neurological or muscular deficits.

In conclusion, the data presented in this study show that α- and β-amyrin, a triterpenoid compound obtained as an isomeric mixture from the resin of *Protium heptaphyllum* exerts pronounced antinociceptive effect in capsaicin models of acute pain and visceral nociception in mice. Although its exact mechanism is unclear, the suppression of C-type fiber activation and an interaction with endogenous opioid system seem likely for the antinociceptive action.

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