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# Determination of pharmacological interactions of uliginosin B, a natural phloroglucinol derivative, with amitriptyline, clonidine and morphine by isobolographic analysis

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## ABSTRACT

Uliginosin B is a natural phloroglucinol derivative, obtained from *Hypericum* species native to South America. Previous studies have shown that uliginosin B presents antidepressant-like and antinociceptive effects. Although its mechanism of action is still not completely elucidated, it is known that it involves the activation of monoaminergic neurotransmission. The aim of the current study was to further investigate the antinociceptive mechanism of action of uliginosin B by combining it with different drugs used for treating pain in clinical practice. The intraperitoneal administration of uliginosin B, morphine, amitriptyline and clonidine, alone or in mixture, produced a dose-dependent antinociceptive effect in the hot-plate assay in mice. The effect of the mixtures of drugs was studied using an adapted isobologram analysis at the effect level of 50% of the maximal effect observed. The analysis showed that the interactions between uliginosin B and morphine was synergistic, while the interactions between uliginosin B and amitriptyline or clonidine were additive. These findings point to uliginosin B as a potential adjuvant for pain pharmacotherapy, especially for opioid analgesia.

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## Introduction

Pain is a direct or indirect common consequence of several diseases. The pharmacological treatment options are extensive and include opioid drugs, non-opioid analgesics (e.g. non-steroidal anti-inflammatory), other classes of drugs (e.g. tricyclic antidepressants and anticonvulsants) and adjuvants (e.g. caffeine and clonidine). The choice of treatment is based primarily on the clinical status. However, the patients with pain, specially moderate to severe, are often under-treated due to lack of effective drugs and/or high rates of adverse effects (Brunton et al., 2007).

Medicinal plants inspired the development of important drugs used in the treatment of pain (such as morphine, acetylsalicylic acid and colchicine) and may still be expected to contribute to the search for new therapeutic strategies as well as new molecular patterns with antinociceptive activity due to chemical diversity (Newman and Cragg, 2012; Phillipson, 2007).

Previous studies performed by our group have shown that uliginosin B (Fig. 1), a dimeric phloroglucinol derivative occurring in *Hypericum* species native to South America (Ccana-Ccapatinta et al., 2014), presents antidepressant-like, antinociceptive and ataxic effects in rodents, depending on the dose range (Stein et al., 2012; Stolz et al., 2012). These effects seem to be at least in part linked to its ability to inhibit neuronal monoamine reuptake (Stein et al., 2012) with consequent activation of dopamine receptors and indirect stimulation of opioid system (Stolz et al., 2012). However, uliginosin B does not bind to the monoaminergic sites on neuronal carriers indicating that it acts differently from the classical antidepressants (Stein et al., 2012). Moreover, uliginosin B does not bind to opioid and dopaminergic receptors and does not stimulate the G protein coupled to these receptors (Stolz et al., 2012).

An alternative to enlarge the pharmacological spectrum is the combination of substances, frequently evaluated through the isobolographic analysis (Tallarida, 2006). The isobolographic analysis is a strategy that allows to investigate the pharmacodynamic and/or pharmacokinetic interaction between two or more substances as well as to study the mechanism of action of these substances (Tallarida, 2007; Wagner and Ulrich-Merzenich, 2009).

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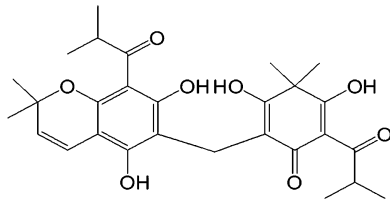


Fig. 1. Uliginosin B structure.

Based on this context, the aim of this study was to further investigate the antinociceptive mechanism of action of uliginosin B through the combination with different drugs used for treating pain in clinical practice, namely morphine (opioid analgesic), amitriptyline (monoamine uptake inhibitor) and clonidine ( $\alpha_2$ -adrenergic and imidazoline receptor agonist) in the hot-plate assay, in mice.

## Material and methods

### Uliginosin B preparation

Uliginosin B was isolated from aerial parts of *Hypericum polyanthemum* Klotzsch ex Reichardt (Guttiferaceae). The vegetal material was harvested during flowering, in Caçapava do Sul (30°31'S; 53°27'W), Rio Grande do Sul state, Brazil, in October 2008. A voucher specimen was identified by Dr. Sérgio Bordignon (UNILASALLE, Brazil) and was deposited in the herbarium of the Universidade Federal do Rio Grande do Sul – ICN *H. polyanthemum*, Bordignon et al. 175915. Plant collection was authorized by Conselho de Gestão do Patrimônio Genético and Instituto Brasileiro do Meio Ambiente – Number 003/2008, Protocol 02000.001717/2008 – 60.

The *n*-hexane extract of aerial parts of *H. polyanthemum* was obtained by maceration, as described elsewhere (Stein et al., 2012; Stolz et al., 2012). Uliginosin B was isolated according to Stolz et al. (2012) and its purity was confirmed through HPLC analysis coupled to an ultra-violet detector by two distinct methods (Ccana-Ccapatinta et al., 2014; Nunes et al., 2009) presenting the peak area greater than 94%; HRESIMS *m/z* 499.2320 [M + H]<sup>+</sup> (predicted for C<sub>28</sub>H<sub>35</sub>O<sub>8</sub>: 499.2332). Uliginosin B was structurally characterized by <sup>1</sup>H and <sup>13</sup>C NMR spectra (60 MHz) without presenting any interfering signal, ensuring the purity of uliginosin B (Parker and Johnson, 1968; Rocha et al., 1995; Taylor and Brooker, 1969).

### Chemicals and drugs

Dichloromethane, *n*-hexane and ethyl acetate were purchased from F. Maia® (São Paulo, Brazil); acetonitrile HPLC, methanol HPLC and deuteriochloroform were purchased from Merck® (Darmstadt, Germany); amitriptyline chloride and clonidine chloride were purchased from Sigma-Aldrich, Co® (St. Louis, USA); morphine sulfate and polysorbate 80 were purchased from Cristalia® (São Paulo, SP, Brazil) and Merck® (Darmstadt, Germany), respectively. All drugs were dissolved in saline (0.9% NaCl) and uliginosin B was suspended in saline with an additional 2% polysorbate 80. All solutions were prepared freshly on the test day and administered by intraperitoneal route (i.p.) at 1 ml/100 g body weight.

### Animals

Adult male CF1 mice (25 – 35 g) were purchased from Fundação Estadual de Produção e Pesquisa em Saúde (Brazil) colony. Six animals were housed per plastic cage (L: 28 cm, W: 17 cm, H: 13 cm) under a 12 h light/dark cycle (lights on at 7:00 h) at constant temperature (23 ± 1 °C) with free access to standard certified rodent diet (Nuvilab CR-1®) and tap water. All experiments were

approved by a local Ethics Committee of Animal Use (UFRGS, number 21060/2011) and were in compliance with Brazilian law (Brasil, 2008, 2013a,b) and Council for International Organization of Medical Sciences International guiding principles for biomedical research involving animals (Bankowski and Howard-Jones, 1985).

### Experimental design and isobolographic analysis

The individual antinociceptive effects of uliginosin B (5, 10, 15 and 90 mg/kg, i.p.), morphine (1, 2, 4 and 8 mg/kg, i.p.), amitriptyline (1, 3, 10 and 30 mg/kg, i.p.) and clonidine (0.03, 0.1, 0.3 and 1 mg/kg, i.p.) were evaluated through the hot-plate assay, performed as described by Stolz et al. (2012). The results were expressed as percentages of maximum possible analgesic effect (%MPE), using the following formula:

$$\%MPE = [(post\text{-}drug\text{treatment}latency - pre\text{-}drug\text{treatment}latency) / (cut\text{-}offlatency - pre\text{-}drug\text{treatment}latency)] \times 100$$

where the cut-off latency was set as 40 s.

Dose – response curves in the hot-plate test were also obtained for the mixtures of uliginosin B with each analgesic drug (morphine, amitriptyline or clonidine) through a fixed-ratio design where the two drugs were co-administered in amounts that keep the proportions of each constant, using multiples of their individual ED<sub>50</sub> values, as follows: 1/8:1/8 (uliginosin B 1.46 mg/kg associated with morphine 0.39 mg/kg or amitriptyline 1.44 mg/kg or clonidine 0.02 mg/kg); 1/4:1/4 (uliginosin B 2.92 mg/kg associated with morphine 0.79 mg/kg, amitriptyline 2.88 mg/kg or clonidine 0.04 mg/kg); 1/2:1/2 (uliginosin B 5.84 mg/kg associated with morphine 1.58 mg/kg, amitriptyline 5.76 mg/kg or clonidine 0.07 mg/kg); 1:1 (uliginosin B 11.67 mg/kg associated with morphine 3.15 mg/kg, amitriptyline 11.51 mg/kg or clonidine 0.14 mg/kg).

The drug-induced motor impairments were assessed by the rotarod test. The animals were treated with the same doses used in the hot-plate test, starting from the highest effective doses. The integrity of motor coordination was established considering the longest time of permanence and the number of falls in a period of 5 min, using the protocol described elsewhere (Stolz et al., 2012). The rotarod test was performed for each mixture at the same doses used in hot-plate test, starting from the highest doses.

The isobolographic analyses (hot-plate test) were performed according to the model described for substances with different maximum effects as described elsewhere (Tallarida, 2006; Woolverton et al., 2008). Firstly the values for ED<sub>50</sub>, maximum effect (*E*<sub>max</sub>) and Hill coefficient (*h*) were determined by non-linear regressions of the dose – response curves (for each drug and mixtures), fixing zero as the minimal effect (*E*<sub>min</sub>). These data were used to calculate the experimental equi-effective doses by the equation:

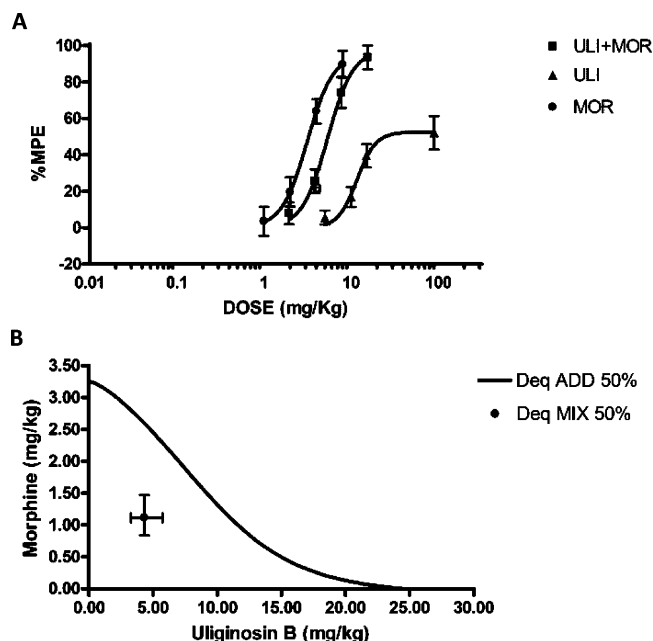
$$E_i = (E_{max}D^h)/(D^h + ED_{50}^h)$$

where *E*<sub>*i*</sub> is the selected effect level (50% MPE) and *D* is the equi-effective dose for this effect level.

The additive line was calculated using the following equation (Grabovsky and Tallarida, 2004):

$$B_i = b + D_B / [(E_B/E_A)(1 + D_A^{h_A}/a^{h_A}) - 1]^{1/h_B}$$

where *B*<sub>*i*</sub> is the equi-effective dose for the drug B alone (morphine, amitriptyline or clonidine) at the level effect (50% MPE); (*a*, *b*) pairs are the doses of drug A and B defining the isobole of additivity; *D*<sub>*A*</sub> and *D*<sub>*B*</sub> are the ED<sub>50</sub> values; *E*<sub>*A*</sub> and *E*<sub>*B*</sub> are the *E*<sub>max</sub> values; *h*<sub>*A*</sub> and *h*<sub>*B*</sub> are the Hill coefficients for drug A and B, respectively.



**Fig. 2.** Analysis of morphine/uliginosin B interaction. (A) Dose – response curves for the antinociceptive effect of morphine, uliginosin B and morphine/uliginosin B combination in the hot-plate test. Data are presented as mean  $\pm$  S.E.M. ( $n=8-10$  mice/group). (B) Isobolographic analysis of morphine/uliginosin B interaction. The continuous line is the line of additivity, representing all the additive theoretical combinations for a 50% effect level. The point (●) represents the experimental combination for a 50% effect level with its 95% CI.

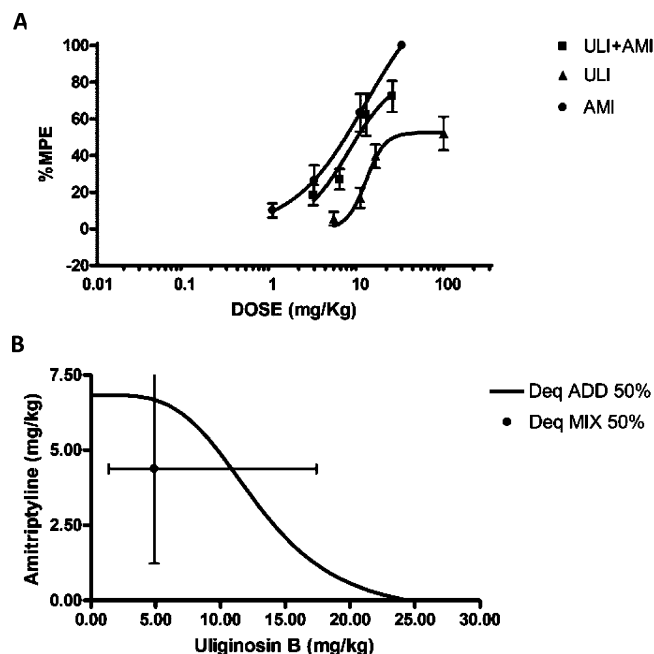
#### Statistical analysis

The rotarod data were evaluated using two-way repeated measures analysis of variance (ANOVA) followed by the Student – Newman – Keuls test (Sigma Stat 3.2 software, Jandel Scientific Corporation®). Differences were considered statistically significant at  $p < 0.05$ . The non-linear regression analyses were performed using GraphPad Prism® version 4.02. All results were expressed in mean  $\pm$  S.E.M or as dose values with their 95% confidence interval (CI).

#### Results and discussion

The intraperitoneal administration of morphine, uliginosin B and mixture of morphine/uliginosin B produced a dose-dependent antinociceptive effect in the hot-plate assay (Fig. 2A). The dose-response curves showed that morphine presents higher efficacy and potency than uliginosin B (Table 1). The isobologram was performed at the experimental doses producing the effect level of 50% (see Table 1). Visual analysis of the isobologram (Fig. 2B) shows that the equi-effective dose pair is below the additive curve, at the chosen effect level (50% of the maximal possible effect), indicating the occurrence of synergism between morphine and uliginosin B. This result is in agreement with previous data showing that the uliginosin B does not bind to opioid receptors and does not stimulate the G protein coupled to these receptors (Stolz et al., 2012) so that its site of action seems to be different from morphine (opioid analgesic), which is compatible with the occurrence of a synergistic effect.

The mechanistic basis of the synergistic interaction may lie in the intricate interplay between opioid and monoaminergic systems. We can speculate that synergism occurs as a result of mutual interaction between independent effects of morphine (opioid receptors activation) and uliginosin B (activation of monoaminergic system). Indeed, several antidepressants that increase the



**Fig. 3.** Analysis of amitriptyline/uliginosin B interaction. (A) Dose – response curves for the antinociceptive effect of amitriptyline, uliginosin B and amitriptyline/uliginosin B combination in the hot-plate test. Data are presented as mean  $\pm$  S.E.M. ( $n=8-10$  mice/group). (B) Isobolographic analysis of morphine/uliginosin B interaction. The continuous line is the line of additivity, representing all the additive theoretical combinations for a 50% effect level. The point (●) represents the experimental combination for a 50% effect level with its 95% CI.

availability of monoamines in the synaptic cleft also exhibit synergism with morphine (Shen et al., 2013).

Stolz et al. (2012) showed that activation of opioid receptors is important for the antinociceptive effect of uliginosin B and suggested that this effect could be the result of increased levels of endogenous endorphins, resulting from the activation of dopaminergic pathways. If we consider this a valid hypothesis, this increase in endorphins activates a subpopulation of opioid receptors which are not activated by morphine, in a similar manner to that demonstrated by other authors, when two opioid drugs have synergistic effect (Miranda et al., 2013).

The results of the rotarod test (Table 2) evidenced that morphine and uliginosin B, when administered alone, induced a motor impairment at doses that promoted the maximum antinociceptive effect, in good accordance with the literature (Loram et al., 2007; Spetea et al., 2010; Stolz et al., 2012). On the other hand, a combination of morphine and uliginosin B that produced the maximal antinociceptive effect (morphine 3.17 mg/kg + uliginosin B 12.35 mg/kg) did not induce motor impairment. This points to uliginosin B as a potential adjuvant to morphine for treating some painful conditions where sedation is not desired.

The intraperitoneal administration of amitriptyline, uliginosin B and mixture of them produced dose-dependent antinociceptive effects in the hot-plate test (Fig. 3A). The dose – response curves showed that amitriptyline presents a higher efficacy than uliginosin B (Table 1); the experimental doses that produced the effect level of 50% (see Table 1) were chosen to perform the isobologram. Visual analysis of the isobologram (Fig. 3B) shows that the equi-effective dose pair is superposed to the theoretical additive curve, indicating the occurrence of additivity between amitriptyline and uliginosin B. The additive effect between amitriptyline and uliginosin B is in line with the fact that both drugs seem to act by enhancing the availability of monoamines in the synaptic cleft (Brunton et al., 2007; Stein et al., 2012).

**Table 1**

Effective doses for the antinociceptive effect of uliginosin B, morphine, amitriptyline and clonidine alone and in combination in the hot-plate test. The ED<sub>50</sub> and the doses producing 50% of the maximum possible analgesic effect (D<sub>50%</sub>) with their respective maximum effect (E<sub>max</sub>) and Hill coefficient (h) were determined by non-linear regression. Doses results are expressed as mean with their 95% confidence interval (CI).

Treatments	ED <sub>50</sub> (95% CI)	D <sub>50%</sub> (95% CI)	E <sub>max</sub> ± S.E.M.	h
Uliginosin B	11.67 (9.03 – 15.07)	24.51 (6.62 – 32.14)	52.52 ± 6.45	4.025
Morphine	3.15 (2.28 – 4.35)	3.26 (2.33 – 4.55)	95.38 ± 12.68	2.950
Amitriptyline	11.51 (2.22 – 59.70)	6.84 (1.86 – 25.06)	136.50 ± 48.06	1.054
Clonidine	0.14 (0.06 – 0.32)	0.13 (0.06 – 0.28)	105.60 ± 18.50	1.430
Morphine/Uliginosin B	5.39 (4.08 – 7.11)	5.44 (4.10 – 7.21)	97.80 ± 10.44	2.993
Amitriptyline/Uliginosin B	7.50 (2.57 – 21.88)	9.29 (2.61 – 32.89)	85.47 ± 35.71	1.615
Clonidine/Uliginosin B	8.03 (2.19 – 29.44)	6.16 (2.12 – 17.86)	133.10 ± 68.15	1.907

**Table 2**

Effect of uliginosin B, morphine, amitriptyline and clonidine, alone and in combination, on the number of falls and permanence time in the rotarod test. Results are expressed as mean ± S.E.M.

Treatments (mg/kg, i.p.)	Fall number		Higher permanence time (s)	
	T <sub>0</sub>	T <sub>30</sub>	T <sub>0</sub>	T <sub>30</sub>
Vehicle	0.8 ± 0.3	1.1 ± 0.4	256.6 ± 11.1	251.5 ± 12.3
Uliginosin B 15.00	1.7 ± 0.6	2.6 ± 0.8	211.4 ± 25.6	187.3 ± 26.9
Uliginosin B 90.00	2.1 ± 0.9	23.4 ± 4.5 <sup>###,***</sup>	230.4 ± 24.4	45.6 ± 14.9 <sup>###,***</sup>
Morphine 4.00	1.9 ± 0.5	1.0 ± 0.5	208.8 ± 25.6	253.2 ± 23.2
Morphine 8.00	0.6 ± 0.2	9.8 ± 2.4 <sup>###,***</sup>	258.9 ± 18.7	131.9 ± 27.4 <sup>###,**</sup>
Amitriptyline 10.00	0.6 ± 0.3	2.2 ± 0.8	250.6 ± 16.6	215.6 ± 26.4
Amitriptyline 30.00	1.2 ± 0.6	64.8 ± 3.2 <sup>###,***</sup>	258.9 ± 18.7	131.9 ± 27.4 <sup>###,***</sup>
Clonidine 0.30	1.5 ± 0.6	0.6 ± 0.4	244.9 ± 21.3	245.5 ± 30.6
Clonidine 1.00	2.5 ± 1.2	18.6 ± 2.0 <sup>###,***</sup>	224.7 ± 23.4	31.8 ± 6.4 <sup>###,***</sup>
Morphine 3.17/Uliginosin B 12.35	1.0 ± 0.3	1.0 ± 0.4	253.7 ± 21.8	244.0 ± 25.0
Amitriptyline 5.51/Uliginosin B 6.18	1.9 ± 0.9	1.3 ± 0.9	243.1 ± 22.0	251.8 ± 21.8
Amitriptyline 11.01/Uliginosin B 12.35	1.9 ± 0.8	20.0 ± 3.8 <sup>###,***</sup>	223.3 ± 25.8	35.1 ± 9.0 <sup>###,***</sup>
Clonidine 0.16/Uliginosin B 12.35	1.7 ± 0.7	1.2 ± 0.4	232.4 ± 19.7	233.7 ± 25.0

<sup>###</sup> Two way repeated measures ANOVA followed by Student – Newmann – Keuls:  $p < 0.001$  difference in relation to T<sub>0</sub> exposition.

<sup>\*\*\*</sup> Two way repeated measures ANOVA followed by Student – Newmann – Keuls:  $p < 0.001$  difference in relation to the same treatment at a lower dose.

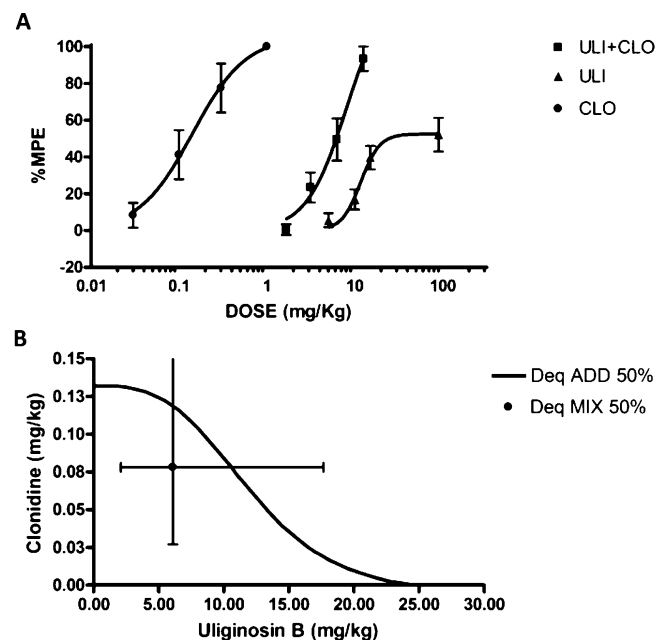
<sup>\*\*</sup> Two way repeated measures ANOVA followed by Student – Newmann – Keuls:  $p < 0.01$  difference in relation to the same treatment at a lower dose.

The results of the rotarod assay (Table 2) indicate that both the substances, when administered alone or in combination, induce a motor impairment at doses that promote the maximum antinociceptive effect, which reaffirms the additivity between these substances. This group of results strengthens the key role of monoaminergic pathways to the mechanism of action of uliginosin B.

The intraperitoneal administration of clonidine, uliginosin B and their mixture produced a dose-dependent antinociceptive effect in the hot-plate assay (Fig. 4A). The dose – response curves showed that clonidine presents higher efficacy and potency than uliginosin B (Table 1); the experimental doses that produced the effect level of 50% (see Table 1) was chosen to perform the isobologram. In this case, the equi-effective dose pair is superposed the additive curve (Fig. 4B), indicating the occurrence of additivity effect between clonidine and uliginosin B.

As uliginosin B appears to act by increasing the availability of monoamines, including noradrenaline (Stein et al., 2012), an opposite effect to clonidine could be expected. The mechanism of action of clonidine is not fully elucidated although it is known that it is an agonist at the  $\alpha_2$ -adrenergic and imidazoline receptors, suppressing the release of noradrenaline in postganglionic sympathetic nerves and that it decreases plasma concentration of noradrenaline (Brunton et al., 2007). In fact, results about the nature of clonidine interaction with monoamine reuptake inhibitors are controversial. Different studies reported that the combination of clonidine with monoamine reuptake inhibitors produces synergistic, additive or antagonistic effects (Eisenach and Gebhart, 1995; Hardy and Wells, 1988; Nikolic et al., 2009).

The rotarod data depicted in Table 2 show that the combination between clonidine and uliginosin B did not impair the motor



**Fig. 4.** Analysis of clonidine/uliginosin B interaction. (A) Dose – response curves for the antinociceptive effect of clonidine, uliginosin B and clonidine/uliginosin B combination in the hot-plate test. Data are presented as mean ± S.E.M. ( $n = 8-10$  mice/group). (B) Isobolographic analysis of morphine/uliginosin B interaction. The continuous line is the line of additivity, representing all the additive theoretical combinations for a 35% effect level. The point (●) represents the experimental combination for a 35% effect level with its 95% CI.

coordination of mice at the dose that present the maximum efficacy in the hot-plate assay contrary to what occurs when administered alone, suggesting that the additive effect does not take place in this behavioral parameter.

In summary, the results so far reinforce the hypothesis that the monoaminergic pathways underlie the action of uliginosin B and point to this natural phloroglucinol derivative as a potential drug to reduce doses of morphine in clinical practice.

### Conflict of interest

No conflict to disclose.

### Acknowledgments

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