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Antinociceptive and anti-inflammatory activities of an aqueous extract of *Chiliotrichum diffusum*

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Abstract: The flowers of the *Chiliotrichum diffusum* (G. Forst.) Kuntze, Asteraceae, have long been used in traditional medicine and rituals. In this study, the anti-inflammatory and antinociceptive activities of a decoction of the flowers were evaluated and a phytochemical analysis was performed by HPLC-DAD. In order to evaluate the antinociceptive activity, the acetic acid-induced abdominal writhing and hot plate tests were used. The anti-inflammatory activity was evaluated using carrageenan-induced rat paw oedema. The decoction induced a significant anti-inflammatory effect (inhibition of 56.0% at 3 h) and produced significant inhibition on nociception in the acetic acid test (ED50 35 mg/kg *i.p.*; ED50 709 mg/kg *p.o*.). In the hot plate test, the antinociceptive activity of the extract employed at 500 mg/kg *i.p*. was significantly suppressed by pretreatment with naloxone (5 mg/kg). HPLC analysis showed the presence of chlorogenic acid, caffeic acid, hyperoside, isoquercitrin, quercitrin, afzelin, quercetin, apigenin and kaempferol. The decoction of *C. diffusum* proved to have antinociceptive and anti-inflammatory effects that may be related to the presence of the flavones, flavonols and phenolic acids identified. The opiod system seems to be involved in the mechanism of antinociception of the extract.

Article

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Introduction

Chiliotrichum diffusum (G. Forst.) Kuntze, Asteraceae, is known with the common name of "mata negra" or "kóor" (Shelkman or Ona´s language) and it is widely distributed in Southern Argentina. It is a medium, branched bush, 50-150 cm high, growing in the Andean region. It is commonly used as food for ovines (Ballaré et al., 2001). This autochthonous specie has had a great impact in the biodiversity patterns in the Andean region, having an appreciable degree of sociability with other species. For these reasons, this species would represent a promissory natural resource for rational exploitation.

The aerial part of this plant has been used in tatoos ("lóiste" in Shelkman or Ona language) in rituals carried out by original people. Besides, the flowers have been traditionally used to "clarify the sight" (Vera, 1991). Although this term is not certainly clear, however it is possible that it is related with an anti-inflammatory activity, since inflammation of the conjunctiva is one of the commonest ocular disorders. Besides, since ancient times, a lot of medicinal plants have been used in ocular ailments (de Brizuela, 2003; Abdul et al., 2010). In this sense, Klauss & Adala (1994) reported that the words employed by African healers to describe eye diseases were mostly related with the inflammatory process.

Other species belonging to the Asteraceae family have proved to possess anti-inflammatory and antinociceptive activities and some of them are also used for eye diseases (Tavares Carvalho, 2004; Dos Santos et al., 2010; Srivastava et al., 2010; Shah et al., 2011). It has been demonstrated that these species possess phenolic acids, such as anthocyanins, flavonols, flavones with high antioxidant activity (Alcalde, et al., 2008), therefore, it is likely that *C. diffusum* also possesses such activities.

Based on these considerations and taking into

account that no pharmacological studies with this plant have been performed so far, the aim of this work was to evaluate the possible anti-inflammatory and antinociceptive activities of the aqueous extract of *C. diffusum*, using classical experimental models of inflammation and pain. A phytochemical analysis was also performed.

Materials and Methods

Plant material

Flowers of *Chiliotrichum diffusum* (G. Forst.) Kuntze, Asteraceae, were collected in summer, in 28 de Noviembre town (Santa Cruz province, Argentina). A voucher specimen was kept in the Herbario Regional Patagónico, Facultad de Ciencias Naturales, Universidad Nacional de la Patagonia San Juan Bosco (number HRP 5305).

Preparation of the aqueous extract

The flowers were dried at room temperature and ground to powder mechanically. The decoction was prepared according to the Farmacopea Nacional Argentina (1978) by pouring 100 mL of cold water onto the 5 g of the ground flowers heating to boiling point and allowing to simmer for 20 min at 100 ºC. The temperature was kept in boiling water bath. The extract was then filtered, to separate the plant residue and the filtered was concentrated using a rotary vaccum evaporator followed by lyophilization. The yield of this extract was 36% (w/w). Before use, the dried extract was dissolved in saline or water for systemic or oral administration, respectively.

Phytochemical studies by HPLC-DAD

The HPLC analysis was performed in a Waters HPLC system consisting of a Waters 1525 Binnary HPLC Pump and Rheodyne (20 µL) injector. The instrument was equipped with a Waters 2996 Photodiodo Array Detector. A Waters symmetric 300 (5 µ) reverse-phase C-18 column (250 x 4.6 mm) was used. As mobile phase, acetonitrile (solvent A) and water-acetic acid (40:1, solvent B) were used. The elution gradient consisted of 15% solvent A and 85% solvent B, for 15 min, it was followed by a linear gradient to 65% solvent B for 30 min and then a sharp transition to 0% solvent B in 2 min. The chromatographic separation was performed at room temperature at a flow rate 1.0 mL.min-1. The elution was monitored at 360 nm. The identification of each compound was achieved by comparing the retention times and UV spectra with those corresponding to commercial standards.

Animals

Twenty-four female Sprague-Dawley rats (180-200 g each), 126 female Swiss mice (20-25 g each) and twenty female and twenty male CF1 mice were used. Animals were housed under standard environmental conditions (22±1 °C and a 12 h light/dark cycle), with free access to a standard commercial diet and water *ad libitum*. Efforts were made to minimize animal suffering. Animals were handled following international guidelines and local regulations concerning the care and use of laboratory animals for biomedical research (Institute of Laboratory Animal Resources, 1996). Experiments with animals were approved by The Ethical Committee for the Care and Use of Laboratory Animals of Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires (Ethics approval: EXP-FYB 0745846/2012).

Drugs

Atropine, indomethacin, lambda carrageenan, morphine sulfate and naloxone were purchased from Sigma Chemical Co (St. Louis, Mo., USA). Acetic acid was purchased from Merck (Darmstadt, Germany).

Antinociceptive activity

Acetic acid-induced abdominal writhing

The test was performed as described by Collier et al. (1968). Nociception was induced by an intraperitoneal (*i.p.*) injection of 1.0% acetic acid, (0.1 mL/10 g body weight). Groups of eight swiss mice were treated with the decoction of the *C. diffusum* either 30 min (10, 30, 100 and 300 mg/kg, *i.p.*) or 60 min (100, 300 and 1000 mg/kg, *p.o.*) before the administration of acetic acid. A group of eight mice was treated with indomethacin (10 mg/kg *i.p.*) as reference drug. Control animals received the same volume of saline solution (10 mL/kg). Animals were observed in experimental cages. In order to assess the participation of different systems on the antinociceptive effect of *C. diffusum*, a separate group of mice was pretreated either with atropine (2 mg/kg *i.p.*) or naloxone (5 mg/kg *i.p.*) 30 min, before the administration of the aqueous extract (30 mg/kg *i.p.*). Hand-operated counters and stopwatches were employed to score the number of abdominal writhes (full extension of both hind paws). The writhes were cumulatively counted over a period of 20 min immediately after the injection of acetic acid. The doses of extract employed had been previously determined by in our laboratory. A significant reduction in the number of abdominal contractions between control and pretreated animals was considered indicative of antinociceptive activity.

Hot-plate test

The hot-plate test was used to measure response latencies according to the method described by Eddy & Leimbach (1953), with minor modifications. Briefly, Swiss mice were placed on an Ugo Basile hot-plate maintained at 56 ºC and the time between placement of the mouse on the platform and shaking or licking any paws or jumping was recorded as the hot-plate latency. Mice with baseline latencies higher than 10 s were eliminated from the study. Twenty-four hours before, the baseline latencies were determined. At the moment of the test, groups of five animals were treated with the decoction of *C. diffusum* (125, 250 and 500 mg/kg *i.p.*), morphine (10 mg/kg, *i.p.*) or saline solution (10 mL/kg, *i.p.*) and left for 30 min. After this period the latencies were determined at 30, 60 and 90 min after each administration. A separate group of mice was pretreated with naloxone (5 mg/kg, *i.p.*) 30 min before the administration of the aqueous extract (500 mg/kg *i.p.*). A significant increase of the latency was considered as indicative of antinociceptive activity.

Anti-inflammatory activity

Carrageenan-induced oedema in rats

Paw swelling was induced by sub-plantar injection of 0.1 mL of 1% sterile lambda carrageenan in saline solution into the right hind paw (Winter et al., 1962). Groups of six Sprague-Dawley rats were used in each treatment.

The decoction of *C. diffusum* was administered at doses of 30 and 100 mg/kg (*i.p.*) 30 min before the injection of carrageenan. Indomethacin (10 mg/kg *i.p.*) was used as reference anti-inflammatory drug. The control group received vehicle only (1 mL/kg *i.p.*). The inflammation was quantified by measuring the volume displaced by the paw, using a plethysmometer (Ugo Basile) at different times after the injection of carrageenan. The difference between the left and the right paw volumes was determined; the degree of inflammation was obtained by the difference between each time (1, 3, 4 and 5 h) and basal time (0 h).

Acute toxicity

Groups of ten CF-1 mice (six weeks old), five male and five female, were used. The control group received only vehicle (water) and the other group received 3 g/kg (0.5 mL/25 g body weight) of the aqueous extract of *C. diffusum p.o.*, by means of a gastric catheter. Animals were observed twice a day, for up to fifteen consecutive days. Apart from the number of deaths, other parameters such as weight loss, abdominal constrictions, palpebral ptosis, movement, lethargy, stereotypy, ataxia, tremors, convulsions, diarrhoea and presence of secretions were taken into account. Dead animals were subjected to necropsy. Liver, lungs, heart, kidneys and stomach were evaluated in order to detect macroscopic changes.

Statistical analysis

Data were presented as the mean±SEM. The statistical significance of differences between groups was assessed by analysis of variance (ANOVA) followed by Dunnett's test for hot plate and acetic acid-induced abdominal writhing tests or Bonferroni test for carrageenan-induced oedema in rats. *p* values less than 0.05 were considered as indicative of significance. Statistical analysis was carried out using statistical package (Graph Pad software, Inc., USA).

Results

Phytochemical analysis

The HPLC analysis of the decoction of *C. diffusum* showed the presence of chlorogenic acid (Rt: 4.57 min), caffeic acid (Rt: 5.54 min), quercetin-3-*O*-β-D-galactoside (hyperoside; Rt: 10.43 min), quercetin-3-*O*-β-D-glucoside (isoquercitrin; Rt: 15.11 min), quercetin-3-*O*-β-D-rhamnoside (quercitrin; Rt: 16.53 min), kaempferol-3-*O*-α-L-rhamnoside (afzelin; Rt: 19.43 min), quercetin (Rt: 30.57 min), apigenin (Rt: 37.01 min), and kaempferol (Rt: 40.37 min). That metabolites were identified in comparison with external standards (Figure 1).

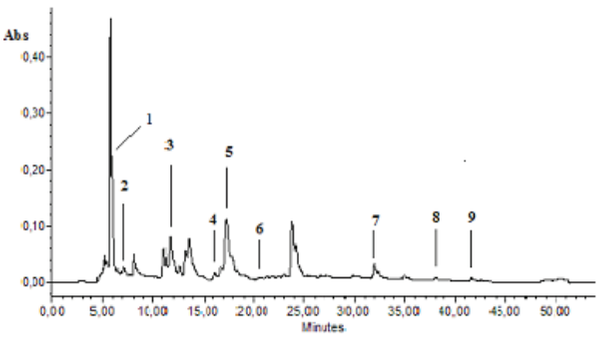

Figure 1. HPLC chromatogram of *Chiliotrichum diffusum* extract. Peaks indicate the following: 1. chlorogenic acid; 2. caffeic acid; 3. quercetin-3-*O*-β-D-galactoside (hyperoside); 4. quercetin-3-*O*-β-D-glucoside (isoquercitrin); 5. quercetin-3-*O*-β-D-rhamnoside (quercitrin); 6. kaempferol-3-*O*-α-L-rhamnoside (afzelin); 7. Quercetin; 8. Apigenin; 9. Kaempferol.

Antinociceptive activity

Acetic acid-induced writhing

The antinociceptive activity of *C. diffusum* was evaluated in the writhing test. The pretreatment of mice with

the decoction (10-300 mg/kg *i.p*. 30 min before stimulus) produced a dose-related inhibition of acetic acid-induced writhing response (ED50 35 mg/kg; 95% confidence limits: 21-51 mg/kg). Moreover, the oral administration of the extract induced a significant inhibition of the writhing response (ED50 709 mg/kg; 95 % confidence limits: 558-921 mg/kg). Indomethacin (10 mg/kg *i.p.*) induced an inhibition of 75.7%. The mechanism of action of *C. diffusum* was investigated by pre-treating animals with drugs that interfere in opiod and cholinergic systems. The pre-treatment with atropine (2 mg/kg *i.p*.) did not modify the antinociceptive response elicited by the extract whereas naloxone (5 mg/kg) significantly prevented the antinociception caused by *C. diffusum* (Table 1).

Table 1. Effect of decoction of *Chiliotrichum diffusum* in the acetic acid-induced abdominal writhing test.

Treatment	Nº writhings	Inhibition (%)
Control	33±3	----
C. diffusum 10 mg/kg *i.p.*	31±5	6.0
C. diffusum 30 mg/kg *i.p.*	12±3**	63.6
C. diffusum 100 mg/kg *i.p.*	7±3**	78.8
C. diffusum 300 mg/kg *i.p.*	4±2**	87.9
C. diffusum 30 mg/kg *i.p*.+atropine	15±4**	54.5
C. diffusum 30 mg/kg *i.p*.+naloxone	25±5$^{\#}$	24.2
indomethacin 10 mg/kg *i.p.*	8±3**	75.7
Control	32±4	
C. diffusum 100 mg/kg *p.o.*	34±4	0.0
C. diffusum 300 mg/kg *p.o.*	21±3*	34.4
C. diffusum 1000 mg/kg *p.o.*	14±3**	56.2

Results were obtained by intraperitoneal (*i.p.*) and oral (*p.o.*) administration of decoction of *C. diffusum.* Each value represents the mean±S.E.M. of results from eight mice. Statistical differences were determined by ANOVA followed Dunnett's test. *p<0.05, **p<0.01 (compared with the corresponding control values injected with vehicle alone); $^{\#}p$<0.05 (compared with *C. diffusum* 30 mg/kg *i.p*. group).

Hot-plate test

The animals treated with morphine (10 mg/kg, *i.p*.) showed a marked increase in latency at all times post-administration whereas the decoction exerted a significant increase in the latency only at 60 and 90 min when the dose of 500 mg/kg *i.p.* was used.

The antinociceptive activity of the extract employed at 500 mg/kg *i.p*. was also significantly suppressed by pretreatment with naloxone (5 mg/kg) (Table 2), confirming the participation of the opiod system in the antinociceptive activity of *C. diffusum*.

Table 2. Effect of decoction of *Chiliotrichum diffusum* in the hot plate test.

Treatment	Latency time (s)		
	30 min	60 min	90 min
control	13.5±1.5	10.0±1.2	8.6±1.0
C. diffusum 125 mg/kg *i.p.*	14.6±2.6	10.5±3.0	9.7±1.5
C. diffusum 250 mg/kg *i.p.*	15.5±0.9	13.2±2.8	12.9±3.2
C. diffusum 500 mg/kg *i.p.*	19.8±1.8	26.5±3.1*	25.9±2.9*
C. diffusum 500 mg/kg *i.p*.+naloxone 5 mg/kg *i.p.*	14.6±2.6	8.7±2.0$^{\#}$	4.5±1.1$^{\#}$
morphine 10 mg/kg *i.p.*	25.2±1.2*	28.3±2.1*	29.0±1.9*

Results were obtained by intraperitoneal (*i.p.*) administration of decoction of *C. diffusum*. Each value represents the mean±S.E.M. of results from five mice. Statistical differences were determined by ANOVA followed Dunnett's test. *p<0.01 (compared with the corresponding control group injected with vehicle alone); $^{\#}p$<0.01 (compared with *C. diffusum* 500 mg/kg *i.p*. group).

Anti-inflammatory activity

Carrageenan-induced oedema test.

The local injection of carrageenan induced a gradual increase in the hind paw oedema volume in the control group. The effect was evident from the first hour after the injection of the phlogistic agent and persisted up to 5 h later with a peak oedema volume being observed after 3 h of the injection. The treatment with indomethacin (the positive control) induced a significant inhibitory effect on paw swelling. The aqueous extract induced a significant anti-inflammatory activity at dose of 100 mg/kg, while the dose of 30 mg/kg did not induce any effect in this model (Figure 2).

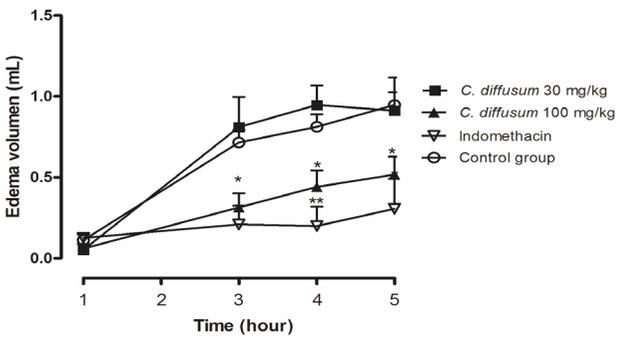

Figure 2. Anti-inflammatory activity of *Chiliotrichum diffusum* in carrageenan induced hind paw oedema. Results were obtained by intraperitoneal administration of decoction of *Chiliotrichum diffusum*, indomethacin and vehicle (control group). Each value represents the mean±SEM of results from six rats. Statistical differences from the control group were determined by ANOVA followed by Bonferroni test. *p<0.05; **p<0.01.

Acute toxicity

The oral LD50 of the decoction was greater than 3 g/kg. After the administration of the extract, no significant difference in body weight was observed between the control and treated group (data not shown). Besides, the extract did not produce any sign of toxicity at the tested dose during the period of observation and at necropsy, no macroscopic changes in organs were detected in the treated groups. These results suggested that the extracts have a low toxicity.

Discussion

The main finding of the present study is that the extract possesses significant antinociceptive activity on models of acute pain induced by a noxious chemical such as acetic acid, as well as by a thermal stimulus. The extract also proved to have anti-inflammatory activity on carrageenan-induced rat paw oedema. The work presented herein represents the first attempt to provide pharmacological evidence of antinociceptive and anti-inflammatory effects of this plant.

The systemic administration of *Chiliotrichum diffusum* (G. Forst.) Kuntze, Asteraceae, reduced pain-related behaviour caused by the administration of acetic acid in the writhing test. The latter is a classical model widely accepted to screen new agents with antinociceptive activity where both neurogenic and/or inflammatory pain are involved. Pain transmission is a complex mechanism that involves interaction of peripheral and central structures and different modulatory pathways (Ossipov et al., 2010). In order to elucidate the mechanisms by which *C. diffusum* exerts its antinociceptive effect in a chemical model of nociception in mice, both cholinergic and opiod systems were evaluated. Although the activity of M1 receptors have been found to increase the pain threshold in a visceral pain model (Ikeda et al., 2001; Wang et al., 2005), the blockade of muscarinic receptors with atropine failed to inhibit the antinociception induced by the extract, indicating that the cholinergic system was not involved in the effect induced by *C. diffusum*. However, the opiod system seemed to be involved, at least in part, in the antinociceptive activity of the extract. Since the algesic effects of acetic acid are due to the release of high levels of mediators such as histamine, serotonin, bradykinin, cytokines and eicosanoids in peritoneal fluid, and knowing that these mediators reduce the threshold of nociception and stimulate nociceptive fibers, it could be hypothesized that the effect of the extract might also be accomplished by a peripheral reduction in the synthesis, of prostaglandins. In order to evaluate different types of pain the hot plate test was used. This is a useful method for the evaluation of central antinociceptive activity, as well as a validated model for opioid-derived antinociceptive compounds. The decoction of *C. diffusum* decreased thermal-induced pain and this effect was reversed by the pretreatment with naloxone, reinforcing the notion that the antinociceptive activity of the aqueous extract of *C. diffusum* is related to the activation of the opioid system.

Since the route of administration is one of the most important factors affecting the results of in vivo methods, the i.p. administration was chosen for primary screening. It is noteworthy that in this study the bioavailability of the extract was quite different in accordance with the route of administration used. Since *C. diffusum* showed the highest antinociceptive activity when administered by the intraperitoneal route, it could be speculated that an extensive hepatic first pass metabolism might be the main reason for the low bioavailability achieved by the oral route.

Further experiments were conducted to investigate a potential systemic anti-inflammatory activity of the extract. The carrageenan-induced paw edema in rats is representative of the inflammatory phenomena occurring during the early stages of an acute process and it is widely accepted as a useful phlogistic tool and highly sensitive test to assess novel anti-inflammatory drugs. The carrageenan injection produces a biphasic event. In the first phase, during the first hour, histamine, serotonin, and bradykinin are the mediators involved, while prostaglandins are implied in the second phase (3-5 h). Results demonstrated that the administration of 100 mg/kg of the decoction of *C. diffusum* induced a significant inhibition of paw edema starting 3 h after carrageenan administration. The latter effect was similar to that obtained with indomethacin, a known nonselective cyclooxygenase (COX) enzyme inhibitor.

In view of the results obtained in the pharmacological assays and taking into account the lack of literature data concerning toxicity, the safety of the decoction was evaluated. The *i.p.* LD50 was greater than 3 g/kg in mice, suggesting that the extracts have a low toxicity.

According to the phytochemical analysis, the presence of different flavonoids such as flavones (apigenin) and flavonols (quercetin, hyperoside, isoquercitrin, quercitrin, kaempferol and afzelin) were identified. Moreover, other sorts of polyphenolic compounds, phenolic acids (chlorogenic and caffeic acids) were found. Some of these compounds have been isolated from other members of the Asteraceae family growing in Argentina, *e.g.* hyperoside, chlorogenic and caffeic acids from *Eupatorium arnottianum* (Clavin et al., 2007).

Among different groups of natural products, the flavonoids are a group of chemical entities widely distributed in the Plantae Kingdom and a great number of pharmacological effects have been ascribed to them (Di Carlo et al., 1999).

Anti-inflammatory and antinociceptive activities

of most of the compounds identified in the decoction have been reported so far. Quercetin and three quercetin derivates (hyperoside, isoquercitrin and quercitrin) (Morikawa et al., 2003; Erdemoglu et al., 2008; Valério et al., 2009), chlorogenic and caffeic acids, natural compounds widely distributed in plants, have previously shown to possess such activities (Morikawa et al., 2003; Dos Santos et al., 2006; Clavin et al., 2007; Erdemoglu et al., 2008; Valério et al., 2009; De Campos Buzzi et al., 2009). During the inflammation process, caffeic acid has shown to be an inhibitor lipoxygenase (Gugliucci et al., 2009), quercitrin and kaempferol-3-*O*-glucoside have proven to be inhibitors of COX-1 and COX-2 (Vanisree et al., 2008). Likewise, apigenin and kaempferol derivatives have exhibited *in vitro* and in vivo activities related to the inflammatory response (Smolinski & Pestka, 2003; De Melo et al., 2009).

Taking into account data presented herein, the presence of compounds identified in the aqueous extract could justify, at least in part, the observed effect.

Although in this work the usefulness of this decoction has not been studied in ocular ailments, it is known that some polyphenols-rich medicinal plants, have been used with this purpose since ancient times (Abdul et al., 2010) and they have also shown anti-inflammatory systemic activity. Therefore, it is likely that this extract might display a beneficial activity on ocular diseases. However, this effect must be subjected to evaluation employing suitable animal models.

The results presented herein demonstrated, for the first time, that the decoction of *C. diffusum* possessed antinociceptive and anti-inflammatory effects that may be related to the presence of flavones, flavonols and phenolic acids. The opiod system seems to be involved in the mechanism of antinociception of the aqueous extract.

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Authors' contributions

SMAB (PhD student) contributed in collecting plant samples and idenfication, running the laboratory work and drafted the paper. MLF participated in chromatographic analysis, drafted the paper and critical reading of the manuscript. OLC contributed to performe the chromatographic analysis and critical reading of the manuscript. CT helped with biological studies. SG contributed with the study design, run the laboratory work, data analysis, drafted the paper and critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

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