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Antinociceptive Orofacial Activity of *Kalanchoe Brasiliensis* Aqueous Extract through Modulation of the TRPV1 Receptor on Zebrafish Model

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Abstract: *Kalanchoe brasiliensis Cambess is a native Brazilian plant that is traditionally used to threat skin, pain, inflammation and gastrointestinal disorders. Here, we evaluated the in vivo orofacial nociception and possible mechanisms of action of the aqueous extract from the leaves of Kalanchoe brasiliensis (ELKB) using adult zebrafish models. The agonist cinnamaldehyde, menthol, capsaicin, acid saline, glutamate, and hypertonic saline were used to evaluate the nociception response of zebrafish, using the open field test to quantify the locomotion activity. In another set of experiments, animals were pre-treated with capsazepine to investigate the mechanism of antinociception. ELKB did not alter the fish's locomotor system and significantly reduced the orofacial nociceptive behavior induced by capsaicin, acid saline, and glutamate compared with the naive group. Capsazepine effectively inhibited the antinociceptive effect of ELKB. This study showed that ELKB has the potential antinociceptive activity by inhibiting the activation of TRPV1, ASICs, and NMDA receptor in adult zebrafish (D. rerio) without affecting locomotion in the animals.*

Keywords: *Kalanchoe brasiliensis; Adult Zebrafish; Orofacial nociception; TRPV1; ASICs; NMDA.*

ABBREVIATION

ASIC	Acid-sensing ion channel
DMSO	Dimethyl sulfoxide
ELKB	Freeze-dried extract Kalanchoe brasiliensis
IASP	International Association for the Study of Pain
ip	Intraperitoneal administration
PBS	Phosphate-buffered saline
NMDA	N-metil-D-aspartato
TRPA1	Transient receptor potential cation channel subfamily A member 1

TRPM8	Transient receptor potential cation channel subfamily M member 8
TRPV1	Transient receptor potential cation channel subfamily V member 1
p.o.	Oral administration

1. INTRODUCTION

Orofacial pain has been described as a lesion or disease of the somatosensory nervous system, often caused by periodontal, musculoskeletal, and neuropathological diseases [1]. Nociceptive stimuli in the orofacial area travel along the trigeminal nerve to higher regions such as the trigeminal nucleus and are modulated on their way to the thalamus, where pain is processed [2,3].

Recently, there has been a surge of interest in the use of zebrafish (*Danio rerio*) for *in vivo* research of pharmaceutical drugs for several diseases once it was proven to be a suitable model with similar morphology and physiology of the nervous, cardiovascular and digestive systems [4]. In addition, some studies have noted the similarity between zebrafish and other vertebrates in relation to the development and organization of peripheral and central nociceptive processing systems and have identified acid-sensing ion channels (ASICs), transient receptor potential cation channel subfamily A member 1 (TRPA1), transient receptor potential cation channel subfamily V member 1 (TRPV1), and glutamatergic nociceptors [(N-metil-D-aspartato (NMDA)] [5].

Kalanchoe brasiliensis is a plant native to Brazil that has recently become of interest to many researchers because of the excellent anti-inflammatory activity of the aqueous or hydroethanolic extract from its fresh leaves [6–9]. Chemically, the aqueous extract contains mainly flavonoids (patuletin class). Among the biological activities investigated in this species are gastroprotective [10], thyroid peroxidase inhibitor [11], acetylcholinesterase inhibitor [12], immunomodulatory [7,13], antibacterial [14,15] and anti-inflammatory activities [6–9,16].

Herein, we evaluated the *in vivo* effects of the freeze-dried aqueous extract from the fresh leaves of *K. brasiliensis* (ELKB) on orofacial nociception using cinnamaldehyde (TRPA1), menthol (Transient receptor potential cation channel subfamily M member 8 - TRPM8), capsaicin (TRPV1), acid saline (ASIC), glutamate (NMDA), and hypertonic saline (TRPV1) in adult zebrafish.

2. MATERIAL AND METHODS

2.1. Plant Material

Fresh leaves of *Kalanchoe brasiliensis* were collected in November 2020 at *Horto de Plantas Medicinais Prof. Francisco José de Abreu Matos* from the *Universidade Federal do Ceará*, Fortaleza, Ceará, latitude 3° 44′ 44.8″ south and longitude 38° 34′ 38.8″ west. A voucher (#EAC0014975) was deposited at the *Herbarium Prisco Bezerra* of the same institution.

2.2. Preparation of the Freeze-Dried Aqueous Extract

The aqueous extract of *K. brasiliensis* leaves was obtained by the method outlined by Costa et al. [17], with minor modifications. Fresh leaves (2.5 kg) were extracted with distilled water using a mechanical crusher for 5 min. Then, the aqueous extract (ELKB) was subjected to centrifugation (Scientific SL-700, Solab, Piracicaba, SP, Brazil) at 1844 x g for 10 min. The decanted juice was transferred to a freezer at -70 °C and then lyophilized (Labconco Kansa City, MO, USA) at -52 °C and 0.018 mBar.

2.3. UPLC-ESI- QTOF/MS Analysis of ELKB

The ELKB was analyzed through a UPLC-ESI-QTOF/MS system using an ACQUITY UPLC BEH column (150 × 2.1 mm, 1.7 µm, Waters Co.) at 40 °C, following the method described previously Carvalho et al. [18]. Briefly, the sample (5 µL) was eluted using a binary gradient elution system consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), with a linear gradient of 98 to 5% B (0-22 min), 5% (22.01-22.50 min), 0% (22.51-24 min), and a flow rate of 0.2 mL/min. The mass spectra were recorded in both positive and negative

ionization modes in a mass range between 110–1180 Da. The source temperature was set at 120 °C, desolvation temperature at 350 °C, desolvation gas flow rate at 500 L/h and capillary voltage at 3.2 kV. The compounds were tentatively identified through molecular formula based on their exact mass (error < 5 ppm) provided by MassLynx 4.1 software, MS fragmentation pattern, chemotaxonomic survey, as well as by comparison with reference standards when available.

2.4. Animals

Adult wild zebrafish (*Danio rerio*) of both sexes (short-fin phenotype), aged 60–90 days, of similar size (3.5 ± 0.5 cm) and weight (0.3 ± 0.2 g) were obtained from Agroquímica: Comércio de Produtos Veterinários LTDA, a supplier located in Fortaleza (Ceará, Brazil). Groups of 50 fish were acclimated for 24 h in a 10-L glass tank (30 × 15 × 20 cm) containing dechlorinated tap water (ProtecPlus®) and an air pump with a submerged filter at 25 °C and pH 7.0 under circadian rhythm (14:10 h of light/dark cycle). The fish were fed *ad libitum* 24 h prior to the experiments. After the experiments, the animals were sacrificed by immersion in ice water (2–4 °C) for 10 min until loss of opercular movements [19]. All experimental procedures were approved by the Ethics Committee on Animal Research of the State University of Ceará (CEUA-UECE), and the protocol was completed under CEUA-02207737/2022.

2.5. General Protocol

The protocols were followed according to Magalhães et al. [20]. On the day of the experiment, zebrafish were randomly selected and then transferred to a wet sponge for treatment with the drugs studied and controls, which were administered orally (*p.o.*) or intraperitoneally (ip). Afterward, they were placed in individual beakers (250 mL) containing 150 mL of water from the fish tank and allowed to recover. In all experiments, an untreated group (naive; n=8) was included.

2.5.1 Locomotor Activity Assessment (Open Field Test)

The animals (n=8) were pretreated with ELKB (1.0, 0.5, 0.25 and 0.125 mg/mL; 20 µL *p.o.*), or vehicle (0.9% saline; 20 µL; ip; control group) and subjected to the open field test as described by Magalhães et al. [20]. After 60 min of the treatments, the animals were then placed in a glass Petri dish (∅ 15 cm) filled with ~50 mL of aquarium water and divided into quadrants. Locomotor activity was analysed by counting the number of crossing lines during 0 - 5 min.

2.5.2 Cinnamaldehyde-Induced Orofacial Nociception

Orofacial nociception was induced with the TRPA1 agonist cinnamaldehyde (Sigma–Aldrich) according to Soares et al. [19]. A solution of cinnamaldehyde (0.33 µM) in DMSO (0.5%) (Labsynth) was applied to the lower lip of the zebrafish (5 µL) 60 min after pretreatment with ELKB (0.5 and 1.0 mg/mL; 20 µL; *p.o.*; n=8). Then, the animals were placed individually in Petri dishes, and the nociceptive response was quantified in terms of locomotor activity for 0 to 5 min.

2.5.3 Menthol-Induced Orofacial Nociceptive Activity In Zebrafish

Orofacial nociception was induced with menthol as described by Soares et al. [19]. Menthol (Sigma–Aldrich) (1.2 mM; 5.0 µL) was injected into the lower lip of the zebrafish (5 µL) 60 min after pretreatment with ELKB (0.5 and 1.0 mg/mL; 20 µL; *p.o.*). Then, the animals were placed individually in Petri dishes, and the nociceptive response was quantified in terms of locomotor activity for 0 to 10 min.

2.5.4 Capsaicin-Induced Orofacial Nociception

Orofacial nociception was induced with the TRPV1 agonist capsaicin (Sigma–Aldrich) (40.93 µM) dissolved in ethanol, PBS (phosphate-buffered saline) and distilled water (1: 1: 8) [19]. Capsaicin was injected into the lower lip of the zebrafish (5 µL) 60 min after pretreatment with ELKB (0.5 and 1.0 mg/mL; 20 µL; *p.o.*). Then, the animals were placed individually in Petri dishes, and the nociceptive response was quantified in terms of locomotor activity for 10 to 20 min.

In a second experiment, zebrafish were pretreated (20 µL; *p.o.*) with ELKB (0.5 mg/mL), vehicle (Control) or capsazepine (Sigma–Aldrich) (TRPV1 antagonist; 30 nM; 20 µL; ip). A fourth group of animals (n = 8) received capsazepine 15 min before ELKB. One hour after the treatments, nociception with capsaicin was induced as described above.

2.5.5 Acidic Saline-Induced Orofacial Nociception

Orofacial nociception was induced with acidic saline [0.1% acetic acid (Dinâmica Química Contemporânea Ltda) dissolved in saline solution (ADV Farma), pH 3.28; 5.0 µL] as described by Soares et al. [19]. The phlogistic agent was injected into the lower lip of the zebrafish (5 µL) 60 min after pretreatment with ELKB (0.5 and 1.0 mg/mL, respectively; 20 µL; *p.o.*). Then, the animals were placed individually in Petri dishes, and the nociceptive response was quantified in terms of locomotor activity for 0 to 20 min.

2.5.6 Glutamate-Induced Orofacial Nociception

Orofacial nociception was induced with glutamate (12.5 µM; 5.0 µL) (Vetec Química Farm Ltda) as described by Soares et al. [19]. Glutamate was injected into the lower lip of the zebrafish (5 µL) 60 min after pretreatment with ELKB (0.5 and 1.0 mg/mL; 20 µL; *p.o.*). Then, the animals were placed individually in Petri dishes, and the nociceptive response was quantified in terms of locomotor activity for 0 to 15 min.

2.5.7 Corneal Nociception Induced By Hypertonic Saline

Corneal nociception was induced with a hypertonic saline solution [5.0 M NaCl (Dinâmica Química Contemporânea)] as reported by Magalhães et al. [21]. After 60 min of pretreatment with ELKB (0.5 and 1.0 mg/mL; 20 µL; *p.o.*), the zebrafish were transferred to a wet sponge, and a drop (5 µL) of 5.0 M NaCl solution was applied locally to the right of the corneal surface. After application, the animals were transferred to Petri dishes. The antinociceptive activity was observed individually for 0-5 minutes.

2.6. Statistical Analyses

The results obtained in this study are expressed as the mean ± standard error of the mean (SEM) for each group of 8 animals. Statistical significance between groups was assessed by one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test. The accepted level of significance for the tests was $p < 0.05$. All tests were performed using GraphPad Prism® v.5 software (San Diego, CA, United States of America).

3. RESULTS & DISCUSSION

Kalanchoe brasiliensis Cambess is a synonym of *K. laciniata* (L.) DC., being native from Brazil and traditionally used to threat skin, pain, inflammation, and gastrointestinal disorders [22]. However, despite of the used in pain, to our knowledge, this is the first study on the orofacial antinociceptive activity of this aqueous extract.

First, after freeze-dry of the ELKB, it was obtained a yellow solid yielding 11.275 g (2.26% w/w fresh plant). The reported yields of ELKB using different ethanol concentrations were 3.44, 4.14, and 4.11 for 30%, 50%, and 70% ethanol in water, respectively [14]. In our study, the yield is less than that reported probably by the freeze-drying process, which produces a drier extract.

It is indispensable to clarify that the chemical composition of the hydroethanolic extract of the leaves of *K. brasiliensis* consists of polar substances, in particular, patuletin-type flavonoid glycosides [9,13,17]. In this study, UPLC-ESI-QTOF/MS chromatograms **(Figure 1)** showed 22 peaks, and it was possible to identify 19 peaks in the negative and positive ionization modes. Among them, among sixteen were patuletin-type flavonoid glycosides [Patuletin-*O*-hexoside-*O*-deoxy-hexoside (Peaks 1a,b; 3a,b; 8a,b); Patuletin-*O*-hexoside-di-*O*-deoxy-hexoside (Peaks 2a,b); Patuletin-O-hexoside-di-O-deoxy-hexoside (Peaks 4a,b; 5a); Patuletin-di-*O*-deoxy-hexoside (Peaks 7a,b); Patuletin-*O*-deoxy-hexoside-*O*-acetyl-deoxy-hexoside (Peaks 13a,b; 17a,b 18a,b); Patuletin-*O*-deoxy-hexoside-*O*-acetyl-rhamnoside (Peaks 14a,b; 15a,b); Patuletin-

di-*O*-acetyl-deoxy-hexoside (Peaks 19a; 20a; 20a,b; 21a)]; two eupafolin-type flavonoid glycosides [Eupafolin-*O*-hexoside-*O*-deoxy-hexoside (Peaks 6a,b); Eupafolin-*O*-deoxy-hexoside-*O*-acetyl-deoxy-hexoside (Peaks 16a,b)], besides the bufadienolide Bryophyllin B (Peaks 12b). The fragment ions at m/z 333 and 317 were identified for aglycones patuletin and eupafolin, respectively [22]. Their retention time, m/z of precursor and fragment ions of the peak along with the respective molecular formulas are detailed in the Supplementary Material.

Our chemical characterization of ELKB is in accordance with previous studies of metabolites found in *K. brasiliensis* [13,17,23,24]. Moreover, the compound patuletin 3-O-α-L-rhamnopyranosyl-7-O-α-L-rhamnopyranoside was observed as the major peak, as shown in **Figure 1** (peaks 7a and 7b). According to Costa et al. [17], this flavonoid is deemed a chemical marker for this plant.

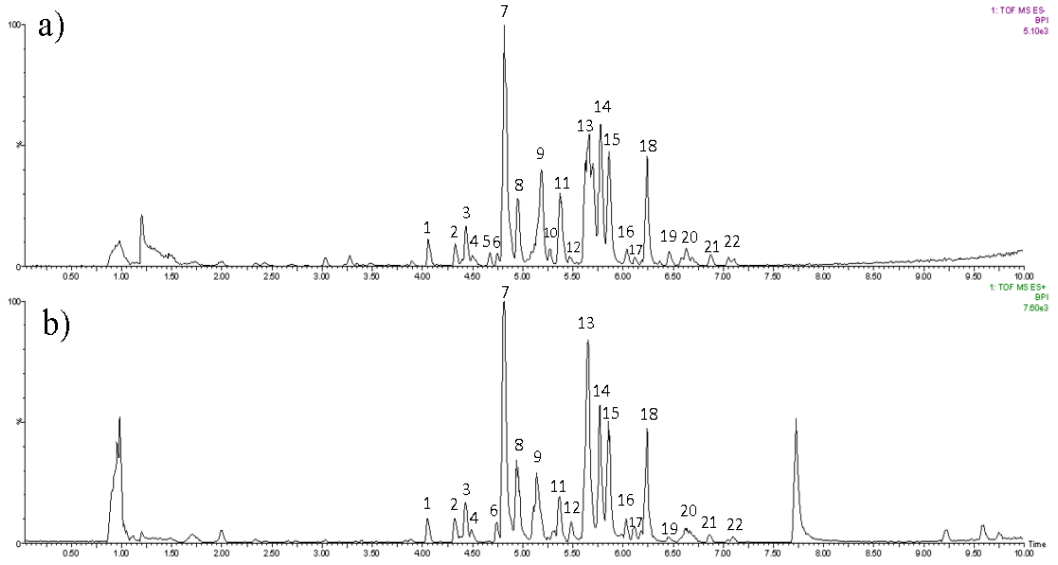

Figure1. *UPLC-ESI-QTOF/MS chromatogram of ELKB in a) negative and b) positive mode*

Previous research in our group has shown that exposure to noxious stimuli can lead to a response in the adult zebrafish behavior when substances that cause pain can reduce the locomotor activity or swimming distance [19–21,25,26]. This is an unfavorable condition for the fish because locomotion is an essential behavior in an animal's repertoire that allows foraging, feeding, escape from a threat, and reproduction, among others [27].

The locomotor activity study, conducted to investigate whether the administration of ELKB (1.0, 0.5, 0.250 and 0.125 mg/mL; 20 µL; p.o.) could alter the locomotion of adult zebrafish in the open field test; this mean, the fish maintained the locomotory patterns as it is to dramatically increased swimming speed/activity; rapid "whirlpool" motion circular swimming; loss of posture and loss of motion for 1–3 s [28], showed that the administration of ELKB did not significantly alter the normal condition of zebrafish, when compared to controls ($p > 0.05$ vs. Control or Naive), as shown in **Figure 2**. Additionally, we report no loss of zebrafish during the assays at the dosed used. The authors Fonseca et al (2018), demonstrated that the hydroethanolic extract of *K. brasiliensis* leaves at doses ranging from 250 to 1000 mg/kg did not exhibit significant toxicity when administered orally to mice, suggesting a safe for use with promising therapeutic potential [24].

Moreover, the phlogistic agents injected into the lower lip or applied to the right eye (hypertonic saline solution) of zebrafish (control group) resulted in a strong decrease in locomotor activity compared to the naive group: cinnamaldehyde ($p < 0.001$ vs. naive), menthol ($p < 0.001$ vs. naive), capsaicin ($p < 0.001$ vs. naive), acid saline ($p < 0.001$ vs. naive), glutamate ($p < 0.05$ vs. naive) and hypertonic saline ($p < 0.001$ vs. naive). The extract ELKB presented an antinociceptive response compared to the control group in three (3) of the six (6) tests performed (**Table 1**).

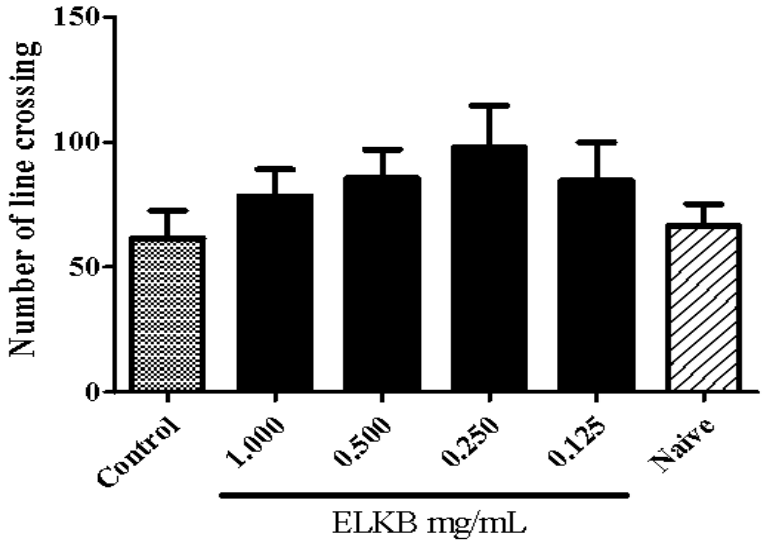

Figure2. *Effect of ELKB on locomotion in adult zebrafish. Results were expressed as mean ± standard error of the mean (SEM) (n=8/group). Differences between groups were analyzed with one-way analysis ANOVA, followed by Turkey's test (*p < 0.05; **p < 0.01; ***p < 0.001; vs control). ELKB: Kalanchoe brasiliensis lyophilized extract; KMC: kalanchosine dimalate salt.*

Table1. *Effect of ELKB on orofacial nociception induced by cinnamaldehyde, menthol, capsaicin, acid saline, glutamate and hypertonic saline in adult zebrafish*

Tx	mg/mL	Groups					
		Cinnamaldehyde	Menthol	Capsaicin	Acid Saline	Glutamate	Hypertonic Saline
		Number of line crossings					
Control	—	20.38 ± 4.87	42.25 ± 9.82	33.38 ± 5.93	92.13 ± 13.11	68.50 ± 11.89	9.75 ± 5.62
ELKB	0.5	29.88 ±6.39	43.50 ± 6.47	**80.13 ± 13.23***	142.80 ± 18.64	67.00 ± 13.17	3.63 ± 2.41
ELKB	1.0	46.50 ± 7.19	64.13 ± 12.02	67.13 ± 9.23	**173.00 ± 15.15***	**86.19 ± 23.15***	6.38 ± 4.99
Naive	—	**67.38 ± 8.89*****	**176.60 ± 17.20*****	**118.00 ± 10.53****	**274.00 ± 29.72*****	**138.10 ± 16.51*****	**59.38 ± 10.07*****

The results are expressed as the mean ± standard error of the mean (SEM) (n=8/group). Differences between groups were analysed with one-way ANOVA followed by Tukey's test (*p < 0.05; **p < 0.01; ***p < 0.001; vs control). Tx: treatment; ELKB: *Kalanchoe brasiliensis* lyophilized extract.

The capsaicin-induced orofacial nociceptive activity**,** results demonstrated that treatment with 0.5 mg/mL ELKB ($p < 0.05$ vs. control) induced an antinociceptive response due to the increase in cross-lining of the zebrafish. Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide—$C_{18}H_{27}NO_3$) the chemical responsible for the burning sensation produced by eating hot peppers, and high proton concentrations like those produced by inflammation [29] and other natural substances as well as by physical factors or protons [30] can activated the transient receptor potential vanilloid 1 (TRPV1) receptor. This activation produces pain by acting on C-fibres and eventually activating free afferent nerve endings that receive noxious stimuli [5,31]. Here, treatments with ELKB prevent the nociceptive activity of capsaicin in zebrafish.

Addition, 1.0 mg/mL ELKB showed antinociceptive activity against acidic saline-induced orofacial nociceptive activity. Acid-sensing ion channels (ASICs) are Na^+ channels gated by extracellular H^+ and are widely expressed in the mammalian central and peripheral nerve systems [32]. A number of substances have been described that can activate the ASIC channel via changes in pH [33]. Acetic acid induces pain in several fish species, including zebrafish, in

which six ASICs have been identified that share up to 75% of amino acid sequences with those in rats and humans [32,34]. Acidic saline is used for this purpose, which has a definite nociceptive effect by activating the ASIC channels when injected into the lip of the fish [19]. Since the upregulation and overactivity of acid sensors appear to contribute to various forms of chronic pain, acid-sensitive ion channels and receptors are considered targets for novel analgesic drugs [35].

Furthermore, treatment with 1.0 mg/mL ELKB demonstrated antinociceptive activity in glutamate-induced orofacial nociceptive activity. Glutamate is an excitatory neurotransmitter involved in the transmission and sensation of pain in vertebrates [36]. It is released peripherally, centrally, and in the spinal cord in response to nociceptive stimulation and tissue or nerve injury [36,37]. Glutamate is critical to several categories of persistent pain, including neuropathic pain resulting from injury and/or disease of central (e.g., spinal cord injury) or peripheral nerves (e.g., diabetic neuropathy, radiculopathy) and inflammatory or joint pain (e.g., rheumatoid arthritis, osteoarthritis) [38]. The modulation of glutamate receptors may have potential therapeutic activity, since this modulation exerts an antinociceptive effect in several mammalian species, including humans [39].

The capsaicin test was chosen to investigate the possible neuromodulation mechanism of ELKB since the lowest concentration had the highest antinociceptive potential in this model. The antinociceptive activity was observed after the treatment with capsazepine ($p < 0.05$) or 0.5 mg/mL ELKB ($p < 0.05$) **Figure 3**. However, when these substances were administrated together, capsazepine significantly prevent the antinociceptive effect of ELKB, showing an antagonistic effect. The authors Soares et al. (2019) [19] demonstrated that the progressive and intermittent use of capsaicin led to an antinociceptive response that is blocked when a TRPV1 receptor antagonist is used, suggesting that ELKB can act on this pathway.

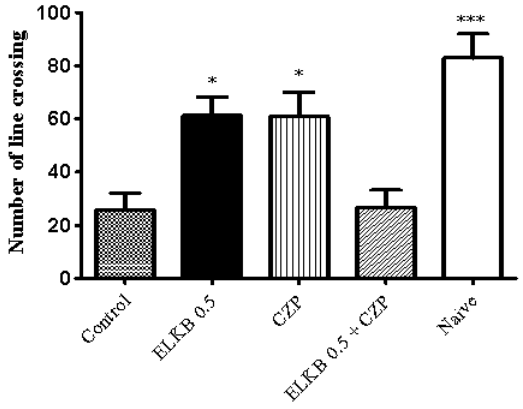

Figure3. *Modulating effect of capsazepine on ELKB induced orofacial nociception in adult zebrafish. Each column represents the mean ± S.E.M. (n = 8/group). ANOVA followed by Tukey test (*p < 0.05; **p < 0.01; ***p < 0.001; vs control). ELKB: Kalanchoe brasiliensis lyophilized extract; CZP: capsazepine.*

Our results are in agreement with the literature, indicating that the antinoceptive activity of the flavonoids presents in ELKB could be related to the pharmacological used of this plant to treat pain disorders, as previously reported by Zarei et al.(2018), who described the effect of the patuletin flavonoid on opioid receptors, modulating the glutamatergic system and inducing antinociceptive effects in male mice [40]. Moreover, Ferreira et al. (2014) reported the antinociceptive activity of flavonoids present in *Kalanchoe pinnata* flowers and leaves, showing the antinociceptive activity through COX inhibition and TNF- reduction [41].

However, treatment with ELKB showed no antinociceptive activity in the cinnamaldehyde, menthol, and hypertonic saline-induced orofacial nociceptive activity.

4. CONCLUSION

This study showed that the aqueous extract from the leaves of *K. brasiliensis* contains patuletin-type flavonoid glycosides as the main metabolites, exhibiting potential antinociceptive activity

by inhibiting the activation of TRPV1, ASICs, and NMDA receptor in adult zebrafish (*D. rerio*) without affecting locomotion in the animals.

SUPPORTING INFORMATION

Their retention time, m/z of precursor and fragment ions of the peak along with the respective molecular formulas of ELKB are detailed in the Supplementary Material.

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SUPPLEMENTARY MATERIAL - Antinociceptive orofacial activity of *Kalanchoe brasiliensis* aqueous extract through modulation of the TRPV1 receptor on zebrafish model

Contents

Table. 1S. Compounds identified by UPLC-ESI-QTOF/MS in negative and positive modes in the extract of the leaves of *Kalanchoe brasiliensis* (ELKB).

Peak no.	Rt min	$[M-H]^-$ Calculated	Product Ions (MS/MS)	Ppm (error)	$[M-H]^+$ Calculated	Product Ions (MS/MS)	Empirical Formula	Ppm (error)	Putative Name	Ref.
1	4.05	639.1561	493.0916; 331.0476; 209.0320; 191.0147; 85.0320	-2.7	641.1718	495.1210; 333.0648; 147.0517	$C_{28}H_{30}O_{17}$	7.2	Patuletin-*O*-hexoside-*O*-deoxy-hexoside	[1]
2	4.32	623.1612	477.1807; 329.02936; 223.0580	-1.8	625.2545	641.1737; 495.1209; 333.0661; 207.0835; 147.0566	$C_{28}H_{32}O_{16}$	---	Patuletin-*O*-hexoside-di-*O*-deoxy-hexoside	[1]
3	4.43	639.1561	477.1033; 331.0383; 329.0303; 153.0925	0.5	641.1824	479.1238; 333.0657; 207.1418; 149.0892	$C_{28}H_{32}O_{17}$	---	Patuletin-*O*-hexoside-*O*-deoxy-hexoside	[1,2]
4	4.51	785.2140	639.1303; 625.1482; 463.0858; 316.0313; 177.0170; 133.187	-3.4	787.1690	641.1659; 495.0968; 481.1026; 333.0676; 319.0493; 147.0638	$C_{34}H_{42}O_{21}$	-4.4	Patuletin-O-hexoside-di-O-deoxy-hexoside	[1]
5	4.67	785.2109	611.2643; 477.1257; 431.1900; 251.1269; 225.0972; 207.1402; 179.0777; 133.0267	---	---	---	---	---	Patuletin-O-hexoside-di-O-deoxy-hexoside	[1]
6	4.74	623.1607	477.1075; 461.1101; 327.0987; 315.0590; 179.0353; 163.0076; 133.0390	2.4	625.1921	463.1512; 317.0713; 191.0976; 147.0667	$C_{28}H_{32}O_{16}$	---	Eupafolin-*O*-hexoside-*O*-deoxy-hexoside	[1,3]
7	4.82	623.1552	477.1055; 331.0444; 315.0126	---	625.1769	479.1245; 333.0663; 129.0566	$C_{28}H_{32}O_{17}$	4.3	Patuletin-di-*O*-deoxy-hexoside	[1,3]
8	4.94	639.1561	477.1023; 331.0452; 316.0205; 163.0260	2.2	641.1849	495.1222; 333.0668	$C_{28}H_{32}O_{17}$	---	Patuletin-*O*-hexoside-*O*-deoxy-hexoside	[1,2]
9	5.18	743.1500	597.0764; 463.0897; 327.0913; 279.0514; 163.0365		609.1938	463.1337; 333.0653; 317.0740; 303.093; 147.0536	---	-7.6	Unknow	---
10	5.25	637.1557	449.2035; 345.0742; 269.1387; 209.1159; 163.0367	-2.7	---	---	$C_{32}H_{30}O_{14}$	---	Unknow	---
11	5.38	559.1147	465.9881; 249.0502; 163.0379; 133.0114; 119.0493; 115.0028;	---	829.2643	683.1963; 495.1273; 333.0699; 147.0502	---	---	Unknow	---

			71.0152							
12	5.46	---	---	---	491.2345	349.1713; 333.0697; 303.1135; 177.0507; 157.0980	---	---	Bryophyllin B	[4]
13	5.65	665.1718	519.1150; 477.1001; 331.0452; 329.0292; 316.0134; 207.0072; 193.0529; 133.0182	-6.5	---	521,1321; 333,0635; 189,0810; 129,0571	$C_{30}H_{34}O_{17}$	4,2	Patuletin-*O*-deoxy-hexoside-*O*-acetyl-deoxy-hexoside	[1-3]
14	5.78	665.1718	477.1014; 331.0482; 329.0292; 315.0120; 207.0108	-5.7	667.1847	479.1233; 333.0638; 189.0807; 129.0568	$C_{30}H_{34}O_{17}$	7.0	Patuletin-*O*-deoxy-hexoside-*O*-acetyl-rhamnoside	[1-3]
15	5.86	665.1718	519.1146; 331.0470; 329.0299; 315.0211; 207.0314; 133.0047	-1.7	667.2027	521.1363; 333.0667; 189.0787; 171.0723; 129.0544	$C_{30}H_{34}O_{17}$	-7.3	Patuletin-*O*-deoxy-hexoside-*O*-acetyl-rhamnoside	[1-3]
16	6.04	649.1856	503.1132; 315.0688; 299.0155; 133.0159	---	651.1953	505.1466; 333.0681; 317.0707	$C_{29}H_{30}O_{17}$	---	Eupafolin-*O*-deoxy-hexoside-*O*-acetyl-deoxy-hexoside	[1]
17	6.11	665.1565	315.0486; 300.0244; 271.0216; 255.0234; 251.1173; 133.0053	2.2	---	---	$C_{30}H_{34}O_{17}$	---	Patuletin-*O*-deoxy-hexoside-*O*-acetyl-deoxy-hexoside	[1]
18	6.24	665.1718	519.1121; 331.0425; 329.0290; 315.0152	1.8	667.1958	521.1374; 333.0668; 129.0582;	$C_{30}H_{34}O_{17}$	---	Patuletin-*O*-deoxy-hexoside-*O*-acetyl-deoxy-hexoside	[1-3]
19	6.46	707.1865	519.1157; 331.1185; 329.0085; 315.0185; 183.0049; 147.0114	---	---	---	$C_{32}H_{36}O_{18}$	---	Patuletin-di-*O*-acetyl-deoxy-hexoside	[1,2]
20	6.63	707.1823	519.1210; 503.1062; 473.2104; 431.2094; 373.2529; 329.0270; 315.0476;	-1.6	---	---	$C_{32}H_{36}O_{18}$	---	Patuletin-di-*O*-acetyl-deoxy-hexoside	[1]
21	6.87	707.1823	519.111; 331.0726; 329.0273; 297.1314; 197.0571; 183.0137	2.3	709.1395	333.0672; 189.0889; 171.0737; 129.0591	$C_{32}H_{36}O_{18}$	4.6	Patuletin-di-*O*-acetyl-deoxy-hexoside	[1,2]
22	7.05	707.1823	519.1221; 331.0124; 329.0389; 297.0934; 197.0253	-3.7	---	---	$C_{32}H_{36}O_{18}$	---	Patuletin-di-*O*-acetyl-deoxy-hexoside	[1,2]