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MITIGATION OF PAIN AND INFLAMMATION-INDUCED IN MICE AND ANTIMICROBIAL ACTIVITY OF CRUDE HYDROALCOHOLIC EXTRACT DE *BEGONIA CUCULLATA* WILLD. (BEGONIACEAE)

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Keywords:

Begonia cucullata, analgesia, Randall-Selitto, Abdominal contortions, Hot plate, Carrageenan-induced edema

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ABSTRACT: *Begonia cucullata* Willd. (Begoniaceae) is a native medicinal species from Paraguay popularly used to combat stomatitis, pharyngitis, respiratory ailments in children, and inflammatory conditions among others. The limited literature did not report these plants as having analgesic, anti-inflammatory, or antimicrobial properties. This work aims to determine the acute toxicity, general behavior, analgesic, and anti-inflammatory activities in mice and antimicrobial activity in-vitro of crude extract of *B. cucullata* (CEBc). Experimental induced-pain models (Randall-Selitto, Writhing, and Hot plate test) were used for the study of analgesia in mice. Likewise, carrageenan-induced paw edema was used to evaluate the anti-edematous activity of CEBc. CEBc is safe, well-tolerated, and without behavioral effects in mice and is devoid of antibacterial activity. Using the Randall-Selitto method, oral doses of CEBc denoted a significant increment in the pain threshold up to 260 %. Also, in the writhing test, the number of abdominal contortions was significantly decreased up to 68% by CEBc in a dose-dependent manner in comparison to the control group. Likewise, oral administration of CEBc in mice presented a 160% increase in latency periods in the hot plate test. Moreover, a 63% reduction in carrageenan-induced paw edema was observed with CEBc treatment. Finally, the presence of alkaloids, tannins, steroids, and/or triterpenes was identified in the CEBc. Based on the results, it is concluded that CEBc has significant analgesic and anti-inflammatory capacity in mice treated orally. These experimental results correlate with popular use in Paraguay and have huge potential for innovation and development.

INTRODUCTION: The major risk for human beings' survival requires the appropriate perception of their environment, effectively identifying hazards, preventing damage to one's body, and promoting effective recovery in the event of damage usually initiated with a painful experience [1].

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Pain is a fundamental experience associated with perceiving actual or potential harm to oneself. Persistent or chronic pain is a public health problem worldwide, and it can result in an

infection, inflammation, peripheral tissue damage, or neural damage [2]. Actually, it is recognized that neurotransmitters, ion channels and neuro-modulators mediate painful responses in the clinic, and in each of the different experimental models of nociception, they may act differently. As a result, either a different drug or a different experimental model will be necessary for a clear explanation of molecular events in attenuating pain [3]. In recent times, a piece of complicated and increasing evidence shows that a large proportion of patients with chronic pain can develop major depressive disorder as comorbidity, in contrast to patients with other chronic medical conditions [4]. Pain and inflammation are pathologies of high prevalence in the world and correspond to one of the gravitating problems in public health. According to WHO reports [5], it affects millions of people in the world, having no limits of age, sex or race with greater impact in vulnerable groups with poor access to medicines. Pain is one of the most relevant symptoms associated with inflammatory disease [6].

Inflammation occurs in vascularized connective tissue and involves vascular changes, cellular events, and the production of chemical mediators of inflammation. All these linked components of the system involve a complicated series of classical events, including arteriolar dilation, increased vascular permeability in venules and capillaries, fluid exudate (including plasma proteins), and migration of leukocytes to the inflamed area [7]. Nowadays is widely accepted among researchers the close relationship between chronic pain and depressive conditions. Indeed, both are very frequent and debilitating concurrent conditions, and they are present in a bidirectional longitudinal relationship due to the high probability of developing depression secondary to chronic pain and vice versa [8]. Nevertheless, approximately a fifth of the patients must end treatment due to adverse effects induced by analgesic drugs, accounting for the urgent development of new drugs devoid of hazards properties [9].

B. cucullata Willd. (Begoniaceae), known commonly as “agrial” is a perennial herb succulent, fibrous-rooted, and fleshy stems that grows in Paraguay, northwestern Argentina, Bolivia and Brazil, among others [10]. The leaves are used for diuresis, treatment of stomatitis, pharyngitis and respiratory ailments in children. Similarly, it is used in bladder inflammation, as a refresher in febrile processes, including those of malaria, and against diarrhea and dysentery. Also, the aerial parts crushed in the ¨tereré¨ (cold water maceration) are used against pharyngitis, stomatitis, and against "mouth ailments". Likewise, the juice of the leaves, applied on the skin is used to heal warts, protruding moles, wounds and inflamed tissues. In addition, the indigenous group maká from the lower Chaco region use a crushed root of this plant into caries to relieve their toothache [11]. The purpose of this pharmacological research is to determine on one hand, the potential in-vitro antibacterial activity. On the other hand, the in-vivo acute toxicity, influence of oral administration of B. cucullata on behavior and its potential analgesic and anti-inflammatory capacity in mice as a measure to validate the use in Paraguayan folk medicine.

MATERIALS AND METHODS:

Plant Material and Extract Preparation: Specimens of *B. cucullata*Willd., (Begoniaceae) were collected in Capiatá. The common name of this plant is agrial. A sample was authenticated (Voucher N: 31 Mirtha González) and was deposited at the herbarium of the Department of Botany (Faculty of Chemical Sciences of National University of Asuncion). Fresh samples of the whole plant were dried in the laboratory environment, cut, and ground until obtaining a fine powder that was subsequently subjected to hydroalcoholic extraction (ethanol: water 70:30) by the conventional reflux method for 1 h. The extraction was repeated 3 times, and the hydroalcoholic extracts were mixed and evaporated under reduced pressure. Finally, the samples thus obtained were used in the biological tests. Also, a preliminary phytochemical analysis of CEBc for the detection of major secondary metabolites was performed using the standard method.

Drugs: All drugs and reagents of analytical quality were used. Sodium chloride, Indomethacin, carrageenan, and others were obtained from Sigma Chemical Company (St. Louis, MO, USA). Diazepam (Roche), pentobarbital sodium (Abbott, Japan), and silica gel plates 60 (F_{254} 20 x 20 cm with 0.20 mm thickness; Merck, Germany) were obtained. Morphine and formaldehyde were

obtained from LASCA Laboratories (Paraguay). Ethanol, propylene glycol, and acetic acid for pharmaceutical use were obtained locally.

Animals: Swiss Albino mice of both sexes (20-30g) were obtained from the animal facility of the Department of Pharmacology at the Faculty of Chemical Sciences. All animals were maintained in room with a controlled environment (23±2 ºC and 55 ± 5 % relative humidity) and with 12 h light/dark cycle. The animals received commercial foods and were fasted overnight before the experiments having free access to drinking water during the trials. All experimental practices performed in the present work were directed in agreement with international animal welfare standards [12]. The protocol was previously approved by the Institutional Ethical Committee in Scientific Research of the Faculty of Medicine on February 09, 2016(CEI-001/16). At the end of the experiments, the mice were submitted to euthanasia by cervical dislocation, frozen and accessible for adequate final disposal of these biological waste.

Pharmacological Assays:

***In-vitro* Antibacterial Activity of CEBc:** The strains were purchased from the American Type Culture Collection (ATCC). Different concentrations of CEBc were tested against a panel of microorganisms including *Escherichia coli* (ATCC 25922), *Escherichia coli* (ATCC 35218), *Staphylococcus aeurus* (ATCC 25923), *Pseudomona aeruginosa* (ATCC 27853), *Enterococcus faecalis* (ATCC 29212), *Klebsiella pneumonia* (ATCC 700603) and awild Strains of microorganisms (*Pseudomonas aeruginosa, Escherichia coli, Citrobacter sp, Staphylococcus aureus*) isolated from hospital.

Inoculum Preparation: The antimicrobial activity was performed using Mueller Hinton agar (MHA), which was distributed in petri dishes (18 mL for each plate) with a thickness of 4 mm. Swabs, sterile Khan tubes, sterile distilled water, filter paper (cut and sterilized), and discs embedded in Amikacin, Tetracycline, respectively, were used.

Disk Diffusion Technique and Minimal Inhibitory Concentration (MIC): The strains were seeded 24 h prior to the test and then, from the grown colonies, suspended in NaCl at 0.9%, corresponding to the 0.5 at McFarland scale (1.5 x 10^8 On CFU/mL), was completed. A suspension of 50 mg of CEBc in 0.5 mL of dimethyl sulfoxide (DMSO) was prepared. The disk diffusion method was performed to evaluate the initial antimicrobial activity of CEBc. The microorganisms were seeded in petri dishes with MHA agar per strain, and 10 µL of the dissolved CEBc was incorporated to the arranged paper discs. All plates were incubated at 35°C for 24 hours and subsequently, the presence or absence of inhibition halos was evaluated [13]. The reproducibility of the technique used was observed carefully. Diffusion discs were impregnated with Amikacin and Tetracycline (for *Enterococcus*) as positive controls, and diffusion discs soaked with the DMSO were used as a negative control. All experiments were performed in triplicate.

Acute Toxicity (LD_{50}) and General Behavior Effect of CEBc on Mice: The oral acute toxicity evaluation was performed in mice using the fixed-dose procedure (FDP) proposed by OECD Guide 425 (Gissi *et al.*, 2017) [14, 16]. Swiss albino female mice (22 - 28 g) fasting overnight were treated with CEBc in a stepwise procedure, using the FDP searching the LD_{50}. The samples were administered orally up to 3000 mg/kg using the FDP method [17]. Two mouse/dose was used in the initial lethality survey search and three mice/dose in the definitive LD_{50} search. After administration, the animals were observed for lethality during the first 24 hours, in the following time intervals 10, 20, 30 and 60 min 2, 3, 4, and 24 hours and daily for 7 and 14 days. After this period, mice were euthanized, and the internal organs were evaluated macroscopically by comparing them with the corresponding organs of the control group. On the other hand, the general behavior of mice was evaluated according to the Irwin procedure [18]. Oral doses of CEBc, ten times lower (30, 100, and 300 mg/kg) than the maximal oral dose used in the LD50 assay (3000 mg/kg), were selected to discriminate the mice behavioral profile induced by CEBc on the peripheral or central nervous system. A mixture of ethanol (10% v/v), propylene glycol (40% v/v), and water (50% v/v), used as a solvent of the CEBc extract, was used as the vehicle.

Determination of the Effect of CEBc on Mice Behavioral Performance (Open Field test): The open field method, as described in the Velázquez *et*

al., (2019) was used. Plastic boxes (40 x 40 x 15 cm) with transparent walls and dark bottoms were used, divided into squares of 10 cm^2 delimited with a line painted in white color. Adult male albino mice (20 - 30 g) were randomly distributed into five different groups (n=6). They were administered with vehicle (0.1 mL/10 g body weight), doses of CEBc (30, 100 and 300 mg/kg, p.o.), and diazepam (0.5 mg/kg, i.p.), respectively. One hour after treatment, all animals sequentially were subjected to 5 min session in the open field behavioral assay to evaluate possible actions on locomotor, exploratory and emotional activities. Diazepam (0.5 mg/kg, i.p.), was used as a positive control and administered 20 min before the trial. Between each record of the behavior of each animal, the open field was cleaned with 10% ethanolic solution to avoid interference from odors typical of stress that can affect the behavior of the animals subjected successively to evaluation. The parameters to be determined are a) the number of quadrants invaded at a fixed time in the periphery (exploratory activity), in the center (anxiolytic or sedative activity), b) self-cleaning (grooming), c) lifting (rearing), d) immobility time (sedation or fear) and e) a number of fecal boluses recorded, respectively for 5 min. In general, central stimulant drugs increase the motor activity of animals, while CNS depressant drugs produce opposite effects [20]. The method also allows for the evaluation of the interaction of depressant drugs or CNS stimulants.

Determination of Pentobarbital-induced Sleep time of Mice Treated Orally with CEBc: Adult albino mice of both sexes (20 - 30 g) were randomly distributed in 5 groups of 5 animals each. The groups were treated with vehicle (0.1 mL/10 g body weight), a dose of CEBc (30, 100, 300 mg/kg, p.o.) and diazepam (0.5 mg/kg *via* i.p.), respectively. After 60 min, each animal was injected with sodium pentobarbital (40 mg/kg i.p.). The group treated with diazepam (0.5 mg/kg, i.p.) received the barbiturate dose after 20 minutes of treatment. This group was considered a positive depressant control as validation of the method. The time in minutes between the injection of the hypnotic and the loss of the posture reflex (induction time) and the time for the spontaneous recovery of the reflex (sleep time) of each animal were recorded [20-22].

Evaluation of the Analgesic Activity of the CEBc in Mice:

Mechanical Pressure-induced Painful Stimulus in Mice (Randall-Selitto Test): Swiss albino female mice (20-35g b. w.) were used and distributed randomly in 5 groups with 6 mice per group. One group was treated orally with the vehicle (0.1 mL for each 10g, b.w.), a second group was treated with Indomethacin (10mg/kg, p.o.), as positive analgesic control, and the other three groups received CEBc (30, 100, and 300 mg/kg, p.o.) dissolved in saline solution. After 60 minutes of the treatments, the reaction to the painful stimulus mechanically induced by caudal pressure, performed with an analgesia meter (LE 306 Panlab, Harvard Apparatus, Spain), was determined (Randall-Selitto Test) [23]. This test was applied increasing the pressure on the midpoint of the tail, previously marked with ink, which is placed between the small base socket and the blunt-tipped piston of the automatically moving arm. The maximum applied force was limited to 250 g to avoid damage to the skin because the animal easily withdraws the tail away upon perceiving the painful stimulus.

Chemically-induced Painful Stimulus in Mice (Writhing test): Swiss albino mice of both sexes (20-30g b.w.) distributed randomly into 5 groups (6 animals/each) were used in this assay. Different groups were treated orally with vehicle (0.1 mL/10g of b.w.), indomethacin (10mg/kg) and doses of CEBc (30, 100 and 300 mg/kg), respectively. After 60 minutes of treatments, 0.8% diluted acetic acid in saline solution was injected intraperitoneally (0.1 mL/10g of b.w.) to all the animals in each group to their corresponding temporal sequences [24]. The number of contortions every 5 minutes was counted for 30 minutes. A contortion was defined as a contraction of the abdominal muscles, elongation of the body, and extension of one or both hind limbs. The means ± standard deviation of the total number of accumulated contortions were registered and related as a function of time in graphs suitable for analysis. The results were expressed as a percentage of analgesia (%AN) according to the following expression:

$$\% AN=100-WE / WC \times 100$$

Where: WE = number of contortions of animals injected with drug, and W C = number of contortions in animals injected with physiological serum

Thermally-induced Painful Stimulus in Mice (Hot Plate test): Swiss albino female mice (25-30 g) distributed randomly into 5 groups (6 animals/each) were used in this assay. The groups were treated sequentially, with the vehicle (0.1 mL/10 g of b. w.; p.o.), a second group with morphine (6 mg/kg, i.p.), and other three groups received CEBc (30, 100, and 300 mg/kg, p.o.), respectively. After 60 minutes and in their corresponding temporal sequences, the animals were placed individually on a hot plate apparatus (56oC) [9].

The animal's reaction time to the thermal stimulus (measured in seconds), characterized by the lifting or licking behavior of the feet, was considered an indicator of the nociceptive effect and the time to be removed from the plate. 30 seconds was considered as the maximum time of contact of the animal with the hot plate. If no reaction is detected, the animal was removed to avoid damage to the feet after this amount of time.

Evaluation of the Anti-inflammatory Activity of the CEBc on Carrageenan-induced Paw Edema in Mice: Swiss albino female mice (25-30 g) distributed randomly into 6 groups (6 animals/each) were used in this assay. The groups were treated, sequentially, with the vehicle (0.1 mL/10 g of b. w.; p.o.), a second group with indomethacin (10 mg/kg, positive anti-inflammatory agent), other three groups received CEBc (30, 100, and 300 mg/kg, p.o.) and one group was used as basal untreated animals, respectively. One hour after receiving treatment, the animals were injected in the sub plantar (s.p.) region of the right hind legs with 40 µL of carrageenan (1%) as described in literature [25].

A similar volume of normal saline solution was injected into the contralateral foot. The paw volume measurements were recorded immediately before carrageenan injection, every 30 minutes, and until completing 3 hours. The procedure consisted of submerging the rear legs to the lateral malleolus into the vessel of the digital plethysmograph (LE 7500 Panlab, Harvard Apparatus, Spain), which measures the volume of the displaced liquid and automatically records the corresponding individual values. The volume difference between the injected legs was considered the final edema value for each animal.

Statistical Analysis: The results were expressed as mean ± standard deviation. The statistical analysis of the data was performed using the analysis of variance (ANOVA) followed by Tukey's multiple comparison test, using the GraphPad Prism 7.0 software. The level $p<0.05$ was considered statistically significant.

RESULTS:

Preliminary Phytochemical Composition of the CEBc: The presence of alkaloids, tannins, steroids and/or triterpenes were identified in the CEBc **Table 1.**

TABLE 1: QUALITATIVE PHYTOCHEMICAL ANALYSIS OF *B. CUCULLATA* EXTRACT (CEBC)

Compounds	Assay	HCl 5%	Chloroform soluble	Chloroform / ethanol	Phenolic alkaloids	Quaternary or amine oxides
Alkaloids	Dragendorff	+	+	+	-	-
	Mayer	+	+	+	-	-
	Valser	+	+	+	-	-
	Reineckate	-	-	-	-	-
Flavonoids	Cyanidin	-				
	HCl 10%	-				
Nafto and/or anthraquinone	Borntrager-Kraus	-				
Tannins	Gelatin-Salt	+				
	$FeCl_3$	+				
Saponins	Hemolysis					
	Foam	-				
Steroids and/or triterpenoids	TLC Lieberman-Burchard	+				

Pharmaco-toxicological tests:

Evaluation of Antibacterial Activity of CEBc: After incubation, diameters of the growth inhibition zone were measured (mm). No significant antibacterial effect against Gram-positive and Gram-negative bacteria was verified at concentrations up to 5 mg of extract in 5 mL of dimethyl sulfoxide (DMSO). The CEBc showed no antimicrobial activity (Data no shown).

Acute Toxicity and General Behavioral Effect of CEBc in Mice: The fixed-dose method proposed by European authorities, agreed upon and accepted worldwide as evidence to determine the safety of chemicals, was used [17, 26]. The oral LD_{50} of CEBc is greater than 3000 mg/kg, in mice of both sexes. In addition, the acute oral doses of CEBc (30, 100, and 300 mg/kg) showed neither signs nor symptoms of poisoning, nor did they cause changes in the general behavioral parameters evaluated consequently the acute oral administration of CEBc is safe in mice.

Evaluation of the Effect of CEBc on Behavioral Performance of Mice Submitted in Open Field Test (OFT): The behavioral performance of male mice submitted to the OFT is shown in **Fig. 1**.

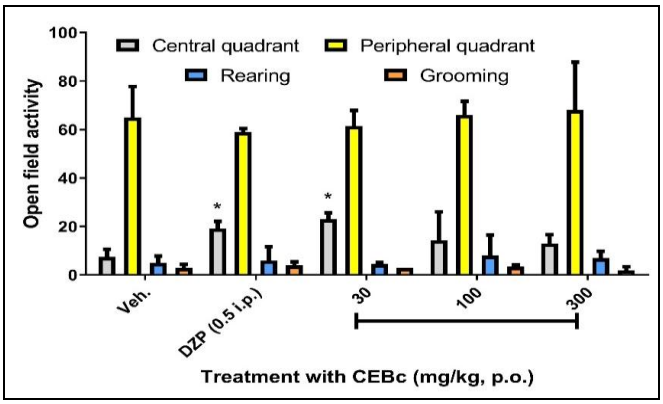

FIG. 1: INFLUENCE OF THE ORAL ADMINISTRATION OF VEHICLE, DIAZEPAM (0.5 MG/KG, I.P.) AND CEBC (30, 100, AND 300 MG/KG P.O.) TO GROUPS OF MICE SUBJECTED TO THE OPEN FIELD TEST. THE BARS REPRESENT THE MEANS ± STANDARD DEVIATION. DIAZEPAM WAS USED AS A POSITIVE CONTROL. THE STATISTICAL ANALYSIS WAS PERFORMED USING ONE-WAY ANOVA FOLLOWED BY DUNNETT'S MULTIPLE COMPARISONS TEST (N=6). * P< 0.05 SIGNIFICANTLY DIFFERENT FROM THE VEHICLE-TREATED GROUP.

The lower dose of CEBc (30 mg/kg; 23.0 ± 2.6; p< 0.05) showed a statistically significant increase in the central quadrant locomotion, compared to the vehicle-treated group (7.4 ± 3.2). Diazepam (19.2 ± 2.9; p< 0.05; positive control) increases the same parameter significantly and validates the method used. Effect of higher oral doses of CEBc (100 and 300 mg/kg) did not modify locomotion, exploratory or emotional behavior.

Effect of CEBc on Pentobarbital-induced Hypnosisin Mice: Groups of animals treated orally with CEBc (30, 100, and 300 mg/kg) did not modify either induction time (data any showed) nor sleeping time of mice submitted to 40 mg/kg (i.p.) of sodium pentobarbital injection in comparison to vehicle-treated group **Fig. 2**. As expected, the positive control (Dzp 0.5 mg/kg i.p.) increased the sleeping time (132.9 ± 19.6), in comparison to the vehicle-treated group (87.8 ± 19.1). This method is considered a very sensitive way to detect agents with depressant or stimulating activity on the central nervous system [20].

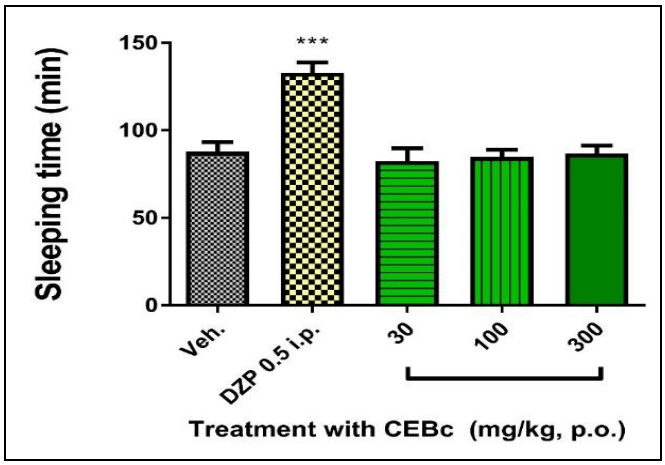

FIG. 2: INFLUENCE OF THE ORAL ADMINISTRATION OF VEHICLE, DIAZEPAM (0.5 MG/KG, I.P.) AND CEBC (30, 100, AND 300 MG/KG P.O.) ONBARBITURATE-INDUCED HYPNOSIS IN MICE. THE BARS REPRESENT THE MEANS ± STANDARD DEVIATION. DIAZEPAM WAS USED AS A POSITIVE CONTROL. THE STATISTICAL ANALYSIS WAS PERFORMED USING ONE-WAY ANOVA FOLLOWED BY DUNNETT'S MULTIPLE COMPARISONS TEST (N=6). *** P< 0.001 SIGNIFICANTLY DIFFERENT FROM THE VEHICLE-TREATED GROUP.

Effect of the CEBc on Mechanical Pressure-induced Painful Stimulus in Mice (Randall-Selitto test): As depicted in **Fig. 3**, the groups of animals treated with doses of 100 mg/kg (690.3 ± 18.4; p < 0,001) and 300mg/Kg (611.8 ± 146.8; p < 0,01) of CEBc, have revealed a statistically

significant analgesic effect in comparison to the group treated with the vehicle (266 ± 229.7). In addition,10 mg/kg of Indomethacin (643.0 ± 159.5; p < 0,01) demonstrated a statistically significant increment in pain threshold effect, which validated the method used. The pain threshold increased by 242, 260, and 230 % in groups treated with indomethacin, 100 and 300 mg/kg of CEBc in comparison to the control group, respectively. Nevertheless, the dose of 30 mg/kg (451.1 ± 169.3), didn't induce a statistically significant response compared to the control group.

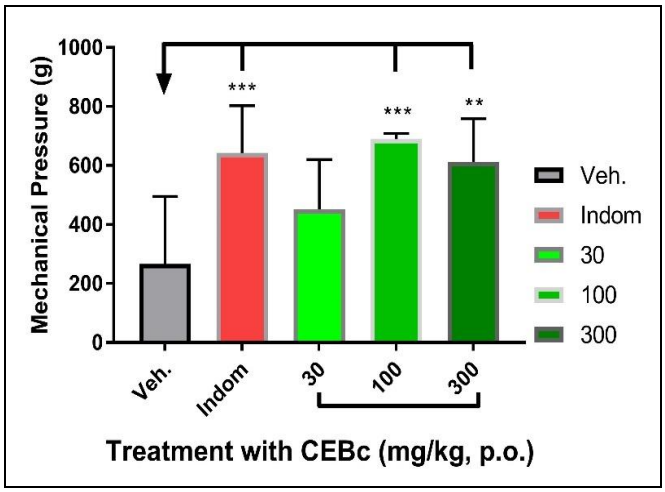

FIG. 3: INFLUENCE OF THE ORAL ADMINISTRATION OF VEHICLE, INDOMETHACIN (10 MG/KG) AND CEBC (30, 100, AND 300 MG/KG P.O.) ON MECHANICAL-INDUCED PAIN IN MICE. THE BARS REPRESENT THE MEANS ± STANDARD DEVIATION. INDOMETHACIN WAS USED AS ANANALGESIC POSITIVE CONTROL. THE STATISTICAL ANALYSIS WAS PERFORMED USING ONE-WAY ANOVA FOLLOWED BY DUNNETT'S MULTIPLE COMPARISONS TEST (N=6). *** P< 0.001; ** P< 0.01 SIGNIFICANTLY DIFFERENT FROM THE VEHICLE-TREATED GROUP.

Effect of the CEBc on Chemically-induced Painful Stimulus in Mice (Writhing test): The groups of animals treated orally with the doses of 30 (43,88 ± 22,67; 51%), 100 (36,50 ± 13,56; 51%), and 300 mg/kg (23,63 ± 8,551; 68%) of CEBc caused a very significant reduction (p<0.001) in the number of abdominal contortions compared to the group treated with the vehicle (77,00 ± 8,32), compatible with an analgesic activity of the sample **Fig. 5**.

In the same sense, administration of Indomethacin denoted a significant reduction of 51% in the number of contortions elicited (30,38 ± 6,63), in comparison to the group treated with the vehicle, validating the utilized method. These results indicated that the dose of 300 mg/kg is more potent than the positive control indomethacin 20 mg/kg in reducing pain behavior induced chemically with acetic acid. The observed effects in this test aren't dose-dependent in nature.

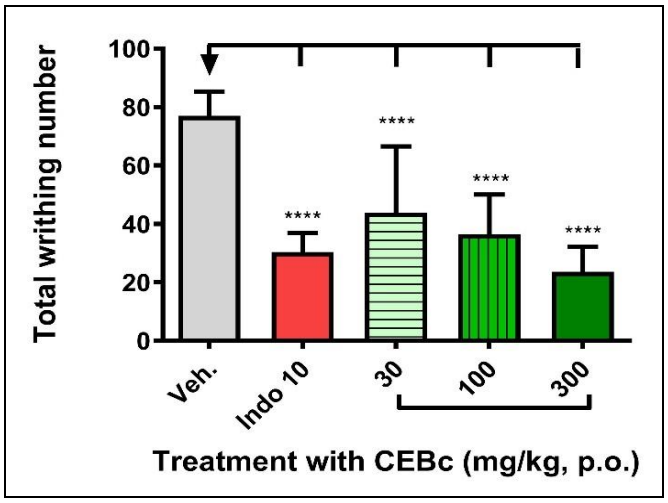

FIG. 4: INFLUENCE OF THE ORAL ADMINISTRATION OF VEHICLE, INDOMETHACIN (10 MG/KG) AND CEBC (30, 100, AND 300 MG/KG P.O.) ON CHEMICALLY-INDUCED PAIN IN MICE. THE BARS REPRESENT THE MEANS ± STANDARD DEVIATION. INDOMETHACIN WAS USED AS AN ANALGESIC POSITIVE CONTROL. THE STATISTICAL ANALYSIS WAS PERFORMED USING ONE-WAY ANOVA FOLLOWED BY DUNNETT'S MULTIPLE COMPARISONS TEST (N=6). **** P< 0.0001 SIGNIFICANTLY DIFFERENT FROM THE VEHICLE-TREATED GROUP.

Effect of CEBc on Thermally-induced Pain Behavior in Mice (Hot Plate Test): As depicted in figure 6, the influence of oral administration of CEBc on reaction time (latency) to heat-induced pain behavior in mice was denoted.

There is a significant increase in the reaction time (pain threshold) of the group treated with 300 mg/kg of CEBc (15.2 ± 2.9 s; p< 0.05; 160%) in comparison to the group treated with the vehicle (9.5 ± 2.6 s). Nevertheless, it should be noted that doses of 30 (13.0 ± 3.1 s) and 100 (10.3 ± 2.2 s) mg/kg of CEBc don't raise the latency time compared to the group treated with the vehicle.

Additionally, a statistically significant increase in morphine-induced latency (14.8 ± 1.3; p< 0.05; 156 %) was observed compared to the negative control group which validates the method used.

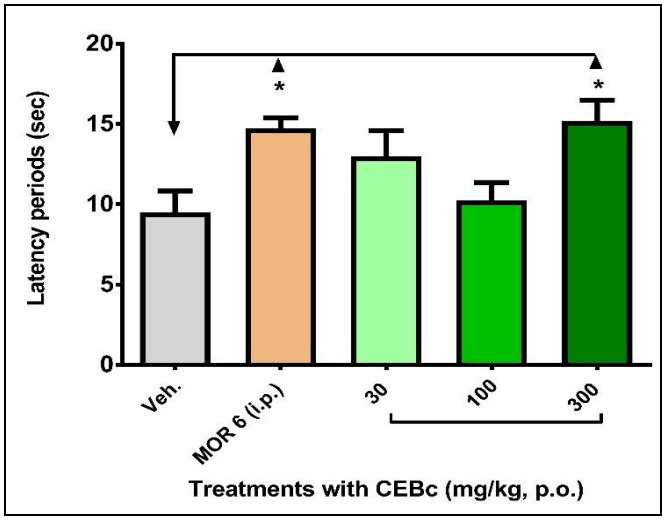

FIG. 5: INFLUENCE OF THE ORAL ADMINISTRATION OF VEHICLE, MORPHINE (6 MG/KG, I.P) AND CEBC (30, 100, AND 300 MG/KG P.O.) ON THERMAL-INDUCED PAIN IN MICE. THE BARS REPRESENT THE MEANS ± STANDARD DEVIATION. MORPHINE WAS USED AS A POSITIVE ANALGESIC CONTROL. THE STATISTICAL ANALYSIS WAS PERFORMED USING ONE-WAY ANOVA FOLLOWED BY DUNNETT'S MULTIPLE COMPARISONS TEST (N=6). * P< 0.05 SIGNIFICANTLY DIFFERENT FROM THE VEHICLE-TREATED GROUP.

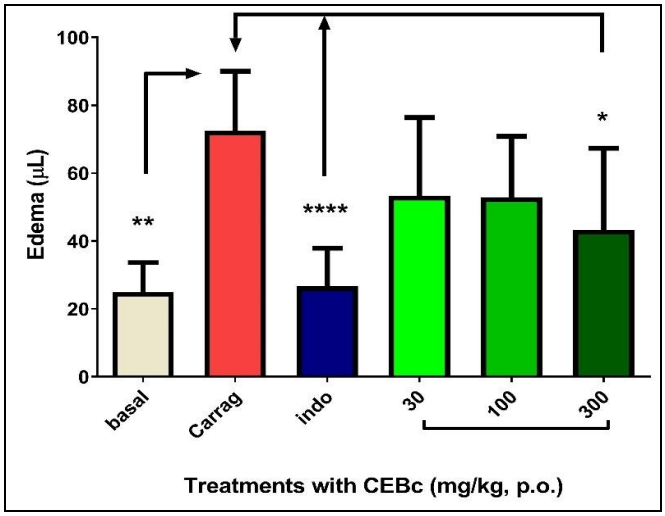

FIG. 6: INFLUENCE OF THE ORAL ADMINISTRATION OF CEBC (30, 100, AND 300 MG/KG P.O.) ON CARRAGEENAN-INDUCED PAW EDEMA IN MICE. THE BARS REPRESENT THE MEANS ± STANDARD DEVIATION. INDOMETHACIN (10 MG/KG P.O.) WAS USED AS A POSITIVE ANTI-INFLAMMATORY CONTROL. THE STATISTICAL ANALYSIS WAS PERFORMED USING ONE-WAY ANOVA FOLLOWED BY DUNNETT'S MULTIPLE COMPARISONS TEST (N=6). * P< 0.05, ** P< 0.01, **** P< 0.0001 SIGNIFICANTLY DIFFERENT FROM THE VEHICLE-TREATED GROUP.

Anti-inflammatory Effect of CEBcon Carrageenan-induced Paw Edema in Mice: Fig. 6 shows the influence of the oral administration of CEBc on carrageenan-induced paw edema in mice. The increased paw edema provoked by 1% carrageenan (72.5 ± 17.5; µL) was reduced significantly in a 40% by oral treatment with 300 mg/kg de CEBc (43.3 ± 24.0; µL; p< 0.05).

In the same sense, the indomethacin 10 mg/kg (26.7 ± 11.2; µL; p< 0.001) provoked a significant decrease of 63% in the inflammatory response provoked by carrageenan, validating the phlogistic method used.

Also, the potency of 300 mg/kg de CEBc effect, represents 62 % of the indomethacin intensity. Nevertheless, it must be noted that groups treated with doses of 30 (53.3± 23.1; µL) and 100 (52.9 ± 18; µL) mg/kg of CEBc didn't significantly reduce the carrageenan induced-edema.

Concurrently, the statistically significant increase of 290 % in the volume of edema was induced by carrageenan (72.5 ± 17.5; µL) in comparison with the untreated basal group (25 ± 8.6; µL) was observed.

DISCUSSION: This study was designed to determine the *in-vitro* and *in-vivo* pharmacological profile of crude extract of *Begonia cucullata* Willd (Begoniaceae) (CEBc), focusing on the antibacterial, antinociceptive and anti-inflammatory activities of this medicinal plant. In addition, in order to examine the safety of CEBc, a preliminary phytochemical study, acute toxicity, and general behavioral assays in mice were performed as preliminary studies. Based on global results, we have shown that CEBc has no antibacterial activity, and orally administered to mice denoted neither acute toxicity nor affected in general behavior, nor central nervous depressant effects. Indeed, for the first time to our knowledge, *B. cucullata* has denoted no antibacterial properties against a panel of gram-positive and gram-negative germs. Thus, its utility in folk medicine in infective conditions (pharyngitis, stomatitis, and "mouth ailments") is potentially not correlated with the experimental *in-vitro* finding. Also, as complementary data, the presence of alkaloids, tannins, steroids and/or triterpenes was identified in the CEBc. On the other hand, in the general *in-vivo* pharmacological evaluation, barbiturate-induced hypnosis was not affected, a moderate significant increase in

locomotor and exploratory behavior in the central quadrant of the open field (OFT) arena was observed in mice treated with CEBc, compatible with a potential anxiolytic effect. Certainly, based on the LD_{50} value, oral administration of CEBc is very safe in mice and potentially is an innocuous medicinal plant for human usage. Based on the International Association for the Study of Pain (IASP) it is considered as "an unpleasant sensory or emotional experience associated with actual or potential tissue damage" and pain has components and their own pathways, centers, and regulatory mechanisms [8].

In general, pain can be classified as: nociceptive, inflammatory, andpathological pain. Nociceptive pain is provoked by the normal activation of peripheral nociceptors characterized by "alarm" and protection of the organism against harmful stimuli, with high threshold pain and often accompanied by a withdrawal reflex. The inflammatory pain appears when invasion of inflammation-mediating cells (macrophages, neutrophil mast cells and granulocytes) is activated by tissue damage and it is characterized by a low pain threshold. The last is the pathological pain (neuropathic and dysfunctional) that appears as a consequence of a lesion to the nervous system (central or peripheral) or abnormal central processing [27]. Behavioral measures of mechanical, chemical, and thermal sensitivity of rodents to pain stimuli, allow knowing the potential analgesic effect of chemicals in the experimental field of pain research using in-vivo accepted biological models [9].

Oral treatment of mice with different doses of CEBc denotes an efficient analgesic and anti-inflammatory capacity when animals are submitted to experimental either nociceptive pain or by carrageenan-induced paw edema models. Using the mechanical pressure-induced pain model (Randall-Selitto test), the pain threshold in mice was increased in a non-dose-dependent manner by 260% and 230% in the groups treated with CEBc (100 and 300 mg/kg) respectively, in comparison to the control group.This fact indicates a major analgesic potential with the medium dose of CEBc (100 mg/kg); while a decreasing degree in the intensity of the analgesic effect was found with the higher dose. The lower dose of CEBc it resulted in a statistically non-significant value. Interestingly, the maximal analgesia induced with the extract was higher 10% in intensity than that obtained with Indomethacin (analgesic reference drug), increasing the pain threshold compared to the control. It should be noted that the behavioral measures of mechanical sensitivity are commonly used to measure allodynia (painful response to non-harmful stimuli) and hyperalgesia. Systems of application of mechanical pressure (caudal, plantar, *etc*.) have been developed to measure pain responses provoked by mechanical stimuli.

Tail withdrawal or vocalization of the animal (fastened) is used as the endpoint of the test. Assessing withdrawal response to pressure applied to tissue, using Randall-Selitto model, reproduces similar results in the reduction of pressure pain threshold, commonly observed in clinical pain conditions (fibromyalgia, myofascial pain, or in osteoarthritis). Likewise, when pain was induced by chemical methods in mice, a strong dose-dependent decreasing sensitivity to pain stimuli (analgesia) was provoked with all doses of CEBc and this effect is strongly compatible with an analgesic activity. This fact may have clinical resemblance and utility for acute pressure pain such as in fibromyalgia [2, 6, 27]. Surprisingly, the intensity of the increased pain threshold observed with the higher dose of CEBc was superior in 22 % than Indomethacin (positive control).

This nociceptive model, chemically-induced, was characterized by acute pain of longer duration (compared to caudal pressure) as a result of peritoneal irritation, involving tissue damage in which inflammatory signaling mechanisms of higher intensity and sensitivity operate. Indeed, the principal mediators in this model are eicosanoids and sympathomimetic amines preceded by the release of TNF-α (nociceptive cytokine) [28]. Similarly, in thermally-induced pain assay, an efficient increase in the latency time either of the hot plate jump or paw lick which is compatible with the analgesic effect of CEBc (300 mg/kg) was observed. The jumping or licking behavior involves a more complex supraspinal structures indicating that this pain stimuli goes beyond nociceptive reflexes because it involves information processing in higher structures [9]. There is a possibility that regulatory pathways mediate the analgesic activity

of CEBc at higher levels because it increases the latency time (increased pain threshold) in the hot plate assay.The use of pain induction procedures allows for evaluating the treatments' effectiveness and analyzing the variables that exert a differentiated effect of the painful experience and constitutes an analogous model to reality. Effectively, the pain threshold increase induced by CEBc in three models is compatible with the analgesic effect. Potentially CEBc may affect, at least partially, either eicosanoids or release of TNF-α (nociceptive cytokine) [28]. However, we have no knowledge about the molecular mechanism of the analgesic action of CEBc or its component. Thus, with the available results, it isn't possible to make any mechanistical hypotheses on main action. Efforts to carry out additional studies to elucidate the regulatory mechanism of the analgesic effect of CEBc are to be reinforced to advance this work.

The inflammatory process constitutes the reaction of a living being to a harmful stimulus. It can be triggered by very diverse harmful agents (infections, antibodies, and physical injuries). The ability to trigger an inflammatory reaction is essential for survival, as the living organism is in contact with environmental pathogens, lesions, or diseases. However, the inflammatory reaction can be too intense and sustained without overt benefit and even carry serious adverse consequences requiring adequate treatment. Diseases that develop with pain and inflammation as part of their symptoms and signs are frequent, which has caused an increase in the consumption of analgesic drugs and nonsteroidal anti-inflammatory drugs (NSAIDs) and steroids (EIGs), leading to an increase of the own adverse effects.

Treatment with medicinal plants, used since ancient times, potentially are an alternative for the relief of these symptoms. However, there is neither enough scientific evidence nor clinical validation of these uses, so it is necessary to research and develop safe and effective anti-inflammatory agents from medicinal plants of traditional use for these conditions. In this context, the higher dose of CEBc was active against the acute inflammation caused by sub-plantar injection of carrageenan in a weaker intensity as induced by Indomethacin. Carrageenan-induced paw edema in the mouse is also associated with nociceptive changes in the paw and migration of inflammatory cells in the injection site and is sensitive to non-steroidal anti-inflammatory drugs [29]. It has been proved lately that the phase of the inflammatory response after the injection of carrageenan in the paw of the mice is associated with the production of the cyclooxygenase 2 (COX-2), high prostaglandin production resulting from cyclooxygenases 1 and 2 (COX-1 and COX-2) activities, free oxygenated radicals like NO (nitric oxide) produced by endothelial and inducible nitric oxide synthase (eNOS and iNOS) and neutrophil infiltration [30, 32]. As demonstrated, CEBc shows anti-inflammatory activity by reducing the edema, but it remains to be determined which of the pathways mentioned above and which component(s) are involved in the analgesia and inflammation. Therefore, this work's results correlate with available literature and validate its popular use as an analgesic and anti-inflammatory treatment.

CONCLUSION: Based on the results, it is concluded that the crude extract of *B. cucullata* is devoid of antimicrobial activity. Also, safe, well-tolerated, without evident central nervous system depressant effects, and with a weak influence on the general behavior of mice. For the first time, the present work demonstrated that the oral administration of CEBc significantly increased the pain threshold in mice submitted to three experimental models of harmful stimuli such as caudal mechanical pressure (Randal-Selitto), intraperitoneal injection of acetic acid (writing test), and heat-induced pain (hot plate), compatible with relevant analgesic activity in equivalent potency as Indomethacin, used as an analgesic and positive anti-inflammatory control.

Moreover, mice submitted to paw edema induced experimentally by carrageenan were reduced significantly by oral treatment to over 62% with a higher dose of CEBc whose potency represents 62% of that caused by Indomethacin (positive anti-inflammatory control). Further, we conclude that the results are correlated with the popular use of CEBc and exposed a variety of possibilities for pharmaco-toxicological investigation and the potential development of innovative phytopharmaceuticals with relevant impact on public health and a huge socio-economic importance. Complementary pharmacological and

chemical works are underway to determine the possible mechanism of action and components involved in the analgesic-anti-inflammatory effect.

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