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Antinociceptive activity of ethanolic extract of *Azadirachta indica* A. Juss (Neem, Meliaceae) fruit through opioid, glutamatergic and acid-sensitive ion pathways in adult zebrafish (*Danio rerio*)

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ABSTRACT

Neem fruit (*Azadirachta indica* A. Juss.) are popularly used to treat infections, diarrhea, fever, bronchitis, skin diseases, infected burns and hypertension. Although the antinociceptive and anti-inflammatory potential of *A. indica* has already been investigated in experimental models of pain and inflammation in mice, the current research is the first to report the evaluation of the capacity of *A. indica* fruit ethanolic extract (EtFrNeem) in acute pain attenuation using the adult zebrafish (*Danio rerio*) as an alternative model to the use in rodents. EtFrNeem was submitted to antioxidant action, preliminary chemical prospecting, FT-IR and determination of phenol and flavonoid content tests. Subsequently, EtFrNeem was tested for acute nociception and abdominal inflammation, locomotor activity, and acute toxicity in adult zebrafish. Possible neuromodulation mechanisms were also evaluated. EtFrNeem showed low antioxidant activity, but was shown to be rich in flavonoids. EtFrNeem showed no anti-inflammatory action, did not alter the locomotor system, and it was not toxic. However, EtFrNeem significantly reduced the nociceptive behavior induced by formalin, glutamate and acidic saline, when compared to the control group. These effects of EtFrNeem were significantly similar to those of morphine, used as a positive control. The antinociceptive effect of EtFrNeem was inhibited by naloxone, ketamine and amiloride. EtFrNeem has the pharmacological potential for acute pain treatment and this effect is modulated by the opioid system, NMDA receptors and ASICs channels. These results lead us to studies of isolation and characterization of EtFrNeem bioactive principles, using adult zebrafish as an experimental model.

Abbreviations: EtFrNeem, *A. indica* fruit ethanolic extract; FT-IR, fourier transform infrared spectroscopy; NMDA, *N*-methyl-D-aspartate (glutamatergic receptor); ASICs, acid-sensing ion channels; eROS, extracellular reactive oxygen species; 3Rs, ethical principle: reduce, refine, and replace; IBAMA-SISBIO, Brazilian Institute for the Environment and Renewable Natural Resources; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); TLC, Thin-layer chromatography; EC_{50}, half maximal effective concentration; TRPA1, transient receptor potential ankyrin-1; TRPV1, transient receptor potential vanilloid 1; PBS, phosphate-buffered saline; TRPM8, transient receptor potential cation channel subfamily M member 8

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1. Introduction

Pain is a common symptom of several chronic diseases, and one of the first signs observed by clinicians, deserving attention and treatment [1,2]. Normally, pain is classified as acute or chronic to differentiate nociceptive from pathological pain, with the latter often being caused by inflammation or neuropathies [3,4]. In general, commercial drugs are used to improve the quality of life of patients with chronic pain, which can result in adverse reactions [5]. Therefore, the search for new drugs as an alternative treatment for pain is a challenge. In this context, extracts obtained from medicinal plants have been reported as being rich in antioxidant compounds, with antinociceptive and anti-inflammatory actions [6]. Such actions are due to the fact that the antioxidant compounds can eliminate the extracellular reactive oxygen species (eROS), originated during the nociceptive processes [7,8]. As such, they constitute a potential source of molecules with better pharmacological activity [9].

Among the several plants rich in antioxidant chemical compounds with analgesic and anti-inflammatory actions, one can mention the Neem (*Azadirachta indica* A. Juss., Meliaceae) fruit. This plant is a perennial tropical tree, native to the Indian subcontinent and also found in Brazil [10,11]. In addition to this potential, it is also used in folk medicine due to its antifungal, antibacterial, antiulcerogenic and antigenotoxic properties [10,12]. The aqueous extract from the stem bark, in addition to its anti-inflammatory action, has been used in folk medicine for its tonic, stimulant, antipyretic, diuretic, as well as antitumoral properties [13]. It is also rich in chemical compounds that show cardiovascular and hypoglycemic action [14]. Neem fruit are popularly used in the treatment of infections, diarrhea, fever, bronchitis, skin diseases, infected burns and hypertension [15].

Seeking bioactive and non-toxic extracts, several natural product research laboratories have employed the use of alternative models to the use of laboratory animals in toxicological tests [16,17]. Among them, we can mention the *in vitro* tests in *Artemia salina* Leach (invertebrate) [18], as well as the adult zebrafish (*Danio rerio*), a vertebrate used in *in vivo* tests [19].

Zebrafish have been used in several studies as an alternative method to the use of rodents, aiming to apply the ethical principle of 3Rs (reduce, refine and replace) [20]. They are used in toxicology tests because they are small, easy to care for, inexpensive to maintain, and produce many transparent embryos that develop outside the parent fish, as well as require a small amount (nanograms) of test sample [21]. They are used in neurobiological tests because they show a human genetic homology of around 70–80% [22]. Recently, they have been used in nociceptive tests [23–25] because their nociceptive physiology is well established and similar to that of mammals [25].

Although the antinociceptive and anti-inflammatory potential of *Azadirachta indica* has already been investigated in experimental models of nociception and inflammation in mice [26–29], this study aimed to evaluate the antinociceptive effect and possible action mechanisms of the ethanolic extract of the *A. indica A. Juss.* (Neem, Meliaceae) fruit using the adult zebrafish (*Danio rerio*) as an alternative model to the use of rodents.

2. Materials and methods

2.1. Collection and identification of plant material

The fruit of *Azadirachta indica* A. Juss. (Neem, *Meliaceae*) were collected, according to the methodology described by Cartaxo et al. [30], in August 2015, in the micro-region of Inhamuns, Tauá, Ceará, Brazil (040°18′05,4″ W; 06°01′03,6″ S), with authorization from the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA-SISBIO) under N. 29145-4. The species was identified and deposited in the Prisco Bezerra Herbarium of the Federal University of Ceará-UFC, exsiccate #56044.

2.2. Drugs and reagents

The following drugs and reagents were used in the study: ethanol (96%; Ciclo Farma). Hexane, Dichloromethane, Acetic acid, Folin–Ciocalteu reagent, and formaldehyde were purchased from Dinamica. Ferric chloride ($FeCl_3$), Sodium Chloride (NaCl), and Dimethyl sulfoxide (DMSO) were purchased from Synth. Formaldehyde (Dinâmica). Saline solution (0.9%; Arboreto). Naloxone (Tocris Bioscience). Diclofenac sodium was obtained from Medley. Gallic acid, amiloride, capsaicin, camphor, carrageenan, Cinnamaldehyde, glutamate, glibenclamide, ketamine, menthol, quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and morphine were purchased from Sigma Aldrich (Brazil).

2.3. Extract preparation

The extract was prepared using the dried fruit (300 g) of *A. indica*, according to Brahequais et al. [31]. The cold extraction was performed using commercial ethanol (96%) as the organic solvent. After 96 h, simple filtrations and total evaporation of the solvent were carried out in a water bath (50 ± 2 °C). The Neem fruit ethanolic extract yield (EtFrNeem) was 75.3 g (25.2%).

2.4. Antioxidant activity

2.4.1. Thin-layer chromatography (TLC) analysis of antioxidant activity

The extract was analyzed by TLC using quercetin and gallic acid as reference standards, as suggested by Hidalgo et al. [32]. The plates were eluted with chloroform/ethanol (9:1) and nebulized with 2.0% $FeCl_3$ or 0.5% DPPH solutions or 1.0% $K_3[Fe(CN)_6]$ and 1.0% $FeCl_3$ [33]. The plates were observed until the appearance of dark blue spots (phenolic compounds) or yellow spots on a purple background (antioxidant compounds) or dark blue or Prussian blue staining (chelating ion reducers).

2.4.2. In vitro antioxidant activity

Solutions of DPPH (3.9 mL, 6.5×10^{-5}M) [34] or ABTS (5 mL; 7 mM) [35] were added to the solutions containing the samples (10 to 10,000 μg/mL). The tests were performed in triplicate. The absorbance values were measured spectrophotometrically at 515 and 734 nm, respectively. The antioxidant capacity was compared with the standard, *i.e.*, quercetin, and EC_{50} was determined.

2.5. Chemical prospecting

2.5.1. Preliminary phytochemical screening and FT-IR analysis

The extract was submitted to preliminary phytochemical screening to detect the main classes of secondary metabolites through chemical reactions that result in changes in color and/or formation of precipitates, which are specific to each class of substances [36].

To identify the possible functional groups that may be present in the sample, a triplicate Fourier transform infrared spectroscopy (FT-IR) was generated using a Shimadzu (Model IR-tracer 100, Japan) spectrophotometer, by employing the standard potassium bromide (KBr) pellet technique [37]. The sample was ground with KBr at a ratio of 1:100. The mixture was placed in the mold and pressed. The pellet was scanned over a wavelength range of 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1} and 64 scans per min. The FT-IR spectrum was expressed as percentage of transmission (%T).

2.5.2. Total phenolic content (TPC) estimation

Folin–Ciocalteu reagent [38] was used with a gallic acid standard curve (1–500 μg/mL). The value obtained from the equation was C = 0.0009 A, where *C* is the concentration of gallic acid, *A* is the absorbance at 750 nm and the correlation coefficient R = 0.9916. The