Research Article

Anxiolytic-like effect of *Azadirachta indica* A. Juss. (Neem, Meliaceae) bark on adult zebrafish (*Danio rerio*): participation of the Serotoninergic and GABAergic systems

Abstract

Azadirachta indica A. Juss is known as the tree of life for its diverse pharmacological applications. This study evaluated the effect of the anxiolytic type of bark of *A. indica* and mechanisms of action on adult zebrafish. Initially, EtCNeem toxicity was detected against *Artemia salina*, eliminated with the saponification reaction. The antioxidant potential of the fractions was evaluated. The fraction with higher antioxidant potential was submitted to the preliminary chemical prospecting, FT-IR, and the phenol and flavonoid content was determined. The animals were treated with the most promising antioxidant fraction (1.0; 2.5 or 5.0 mg/mL, *i.p.*) and underwent acute toxicity, open field tests and Light & Dark Test, evaluating the participation of serotonergic and GABAergic systems in the anxiolytic test. As a result, we selected the fraction of ethyl acetate (F-EtOAc) rich in phenolic and flavonoid compounds with antioxidant potential, which had a sedative and anxiolytic effect on adult zebrafish through serotonergic and GABAergic systems. F-EtOAc prevented alcohol-induced chronic anxiety in adult zebrafish. The results suggest that F-EtOAc is an anxiolytic agent mediated *via* the GABAergic and serotonergic system.

Keywords: neem barks, acute and chronic anxiety, adult zebrafish, $GABA_A$, 5-HT

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Introduction

Anxiety disorders are among the top ten diseases responsible for disability worldwide, and Brazil, in 2015, was considered by the World Health Organization (WHO) the country with the highest rate of anxiety disorders in the world, with 9.3% of the population.[1]

Abstinence-induced anxiety is a common problem in drug abuse.[2] This problem is associated when consumption is discontinued or abruptly reduced, with the occurrence of symptoms such as trembling, anxiety, insomnia, agitation, hypervigilance, irritability, piloerection and, sometimes, seizures.[3] Benzodiazepines (GABA receptor agonists) and selective serotonin reuptake inhibitors (SSRIs) are the drugs of choice for the treatment of anxiety.[2] SSRIs are also commonly used to treat depressive disorders.[4] However, the chronic use of benzodiazepines causes tolerance, and abrupt treatment discontinuation may lead to withdrawal syndrome.[5] On the other hand, the chronic use of SSRIs can result in considerable side effects,[6] therefore the search for new compounds with anxiolytic and antidepressant properties that cause fewer adverse effects continues.[7]

Animal models are used for the assessment of new anxiolytic drugs. These pre-clinical models and screening tests support the studies, since clinical trials are expensive,[8] especially regarding the central nervous system therapy.[9] Currently, the zebrafish (*Danio rerio*) has been used in behavioral neuroscience, including research involving the brain and psychopharmacology.[9] This vertebrate animal is considered a significant model in preclinical studies, because its genotype has 70% homology with mammalian neurotransmitter receptors.[10]

Because of the side effects associated with allopathic drugs, there is a growing interest in the development of alternative therapies to treat psychiatric disorders.[11] Previous studies have demonstrated the anxiolytic action of several phytochemical groups, such as polyphenols and flavonoids found in plants.[12] In this context, *Azadirachta indica* A. Juss. (Neem), which belongs to the Meliaceae family, native to India, and also found in Brazil, is rich in flavonoids.[13] Both the bark and leaves of *A. indica* contain biologically active molecules, which have hypolipidemic, hypoglycemic, immunostimulatory, hepatoprotective,

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anti-inflammatory and antifertility properties.[14] *A. indica* limonoids and phytosterols play an important role, having anti-inflammatory, immunomodulatory and antioxidant properties.[15] Previous study carried out with *A. indica* leaf extracts have demonstrated anxiolytic action in rats.[16]

Based on the abovementioned facts, this study aimed at evaluating the anxiolytic-like effect of *A. indica* A. Juss bark and possible mechanisms of action in adult zebrafish (*Danio rerio*).

Material and methods

Drugs and reagents

The following reagents and drugs were used in the study: commercial ethanol (96%), ethyl alcohol PA (99.5%; Biotec®), KOH (99% m/m; Vetec®), HCl (36.5% v/v; Microquímica®); Ferric chloride, gallic acid, KBr, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Tween 80, flumazenil (Sandoz), granisetron hydrochloride (Kytril), dichloromethane, ethyl acetate, Folin-Ciocalteu, pizotifen maleate (Sandomigran®), fluoxetine (Teuto), Cyproheptadine (Cobavital®) and Diazepam (Teuto).

Botanical material

The *A. indica* bark was collected in the city of Tauá (040°18'05,4" W; 06°01'03,6" S) state of Ceará, Brazil, after obtaining authorization from SISBIO, according to the registration for the collection of botanicals, fungal and microbiological material, n° 29145-4. The botanical identification was carried out in the Prisco Bezerra Herbarium of the Federal University of Ceará, where an exsiccate was deposited under number 56044.

Extract preparation

The crude extract was obtained from the bark (350g) of *Azadirachta indica* (Neem, Meliaceae), using the methodologies described by Matos.[17] Commercial ethanol (96 °GL) was used as the organic solvent, and after 96 hours of cold extraction, simple filtrations were performed, and the extracts were kept in a water bath (50±2°C) for complete solvent evaporation. The yield of the obtained Neem bark ethanolic extract (EtCNeem) was 89.4 g (25.54%).

Saponification reaction

The saponification reaction was performed following the methodology proposed by Scroder.[18] EtCNeem (5 g) was dissolved in an alcoholic solution (ethyl alcohol P.A. 99.5% Biotec®) of Vetec KOH (99%; m/m). A thermostatic water bath was used at 60ºC for 60 minutes for the saponification reaction of the sample fatty acids. After the completion of the reaction, the material was transferred to an ice bath and after it cooled, 36.5% HCl (pH 1.0) was added for complete release of the fatty acids from their respective salts. The unsaponifiable organic compounds were extracted using dichloromethane and ethyl acetate, then separated from the residual fraction (saponifiable compounds or fatty acids) through the settling funnel. The yields of the unsaponifiable fractions dichloromethane (F-Dic) and ethyl acetate (F-EtOAc) obtained were 2.56 g (10.24%) and 0.62 g (2.48%), respectively. A yield of 21.82 g (87.28 %) was obtained from the saponifiable fraction (F-Sap).

Antioxidant activity-DPPH Free Radical Scan

A methanol solution of DPPH was added to the methanolic solutions of the samples (10 to 10.000µg/mL). The test was performed in triplicate. After the 60-minute interval, absorbance was measured in a UV-Vis spectrophotometer at 515 nm. Pearson's correlation (*r*) was used to evaluate the association between phenol and flavonoid contents with antioxidant activity. The antioxidant capacity was compared with a standard quercetin curve and EC_{50} was determined.[19]

Preliminary phytochemical screening

The fraction with the best antioxidant activity (F-EtOAc, see Results section) was submitted to a preliminary phytochemical screening to detect the major classes of secondary metabolites through chemical reactions that result in color changes and/or result in the formation of a precipitate specific to each class of substances, using the methodologies described by Matos.[17]

Fourier Transform Infrared Spectroscopy (FT-IR)

Fourier Transform Infrared Spectroscopy (FT-IR) was performed to identify the possible functional clusters present in F-EtOAc. The FT-IR data were obtained using an IRTracer-100 Spectrometer (Shimadzu, Japan), in which 2 mg of the sample was dispersed in 200mg of KBr and pressed at 80 kN to form the KBr pellets, and then analyzed in the range of 400-4000 cm^{-1} with a resolution of 4 cm^{-1} and 64 scans per minute.

Measurement of the total phenolic compounds

The measurement of the total phenolic compounds of F-EtOAc was performed using the Folin-Ciocalteu method,[20] where a calibration curve was prepared using diluted solutions of gallic acid (0-500µg/mL). The total phenol (TP) content was determined by interpolating the absorbance of the samples against a calibration curve constructed with gallic acid standards and expressed as mg of EGA (equivalent of gallic acid) per 100 g of extract. The equation for the gallic acid calibration curve was C=0.0009A, where C represents the concentration of gallic acid, A represents the absorbance at 750 nm and the correlation coefficient R=0.9916. The TP means, and the respective standard deviations were submitted to analysis of variance (ANOVA) using the software *GraphPadPrim*. Significant differences (*p*<0.05) between the means were determined by *Tukey* multiple comparison test.

Estimation of total flavonoids content

The total flavonoids content of the extract was analyzed utilizing aluminum chloride (AlCl3) method.[21] The calibration curve was prepared with quercetin [500–31.25µg/mL in ethanol at 80 % (v/v)]. Samples of 0.5 mL of each extract (1 mg/mL) or standard solutions were united with 1.5 mL of ethanol at 95 % (v/v), 0.1 mL of AlCl3 at 10 % (w/v), 0.1 mL of sodium acetate at 1 mol/L and 2.8 mL of distilled water. In the blank test, the volume of aluminium chloride at 10% was substituted by distilled water. The incubation occurred at room temperature for 30 min and the absorbance reading was measured at 415 nm in a spectrophotometer. The measures were performed in triplicate and the average value was expressed in µg of quercetin equivalents (QuerE) per milligrams of extract (µg/mg).

Toxicity to Artemia salina

The solutions of EtCNeem and fractions (100, 500 and 1000µg/mL) were tested in triplicate. The surviving nauplii were counted after 24 hours and the percentage of dead nauplius was determined by calculating the lethal concentration that will kill 50% (LC_{50}) using the Probit analysis with 95% confidence. The toxicity potential of the substances was classified as: a) Non-toxic (LC_{50}> 1000µg/mL); b) Toxic (LC_{50} ≤ 1000µg/mL).

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Adult Zebrafish (Danio rerio) (aZF)

Wild adult zebrafish (60 to 90 days old; 0.4±0.1 g, 3.5±0.5 cm), of both genders, were obtained from a commercial supplier (Fortaleza, CE). Groups of animals (n = 30) were acclimatized for 24 h in a 10 L glass tank (30 × 15 x 20 cm) containing dechlorinated tap water (ProtecPlus®) and a submersible filter and air pump at 25°C and pH 7.0, under a circadian cycle of 14:10 h (light/dark). Animals received *ad libitum* feed 24 h prior to the experiments. After the experiments, the animals were euthanized by immersion in ice water (2-4°C) in up to 2 minutes until loss of opercular movements occurred. All experimental procedures were approved by the Animal Research Ethics Committee of the State University of Ceará (CEUA-UECE # 7210149/2016).

Acute toxicity to adult zebrafish

The study was carried out based on the methodology proposed by Ekambaram et al.,[22] with adaptations. As an adaptation of the method, aZF (n=6/group) were treated intraperitoneally (*i.p.*) with 20μL of the best antioxidant fraction (1.0; 2.5 and 5.0 mg/mL) of non-toxic samples against *A. salina* (see Results section). As negative control, 3% Dimethyl sulfoxide (DMSO) (20μl, *i.p.*) was used. After 24, 48, 72 and 96 hours, the values obtained regarding the number of dead aZF were submitted to statistical analysis, estimating the lethal dose to kill 50% (LD_{50}) of aZF utilizing the *Trimmed Spearman*-Karber method with 95% confidence intervals.

Evaluation of locomotor activity

The Open Field Test proposed by Magalhães et al.,[23] was used to evaluate whether or not there was alteration of the motor coordination in the animals. aZF (n=6/group) were treated intraperitoneally (*i.p.*) with F-EtOAc (1.0 or 2.5 or 5.0 mg/mL, 20μL; *i.p.*) or vehicle (DMSO 3%; *i.p.*) or diazepam (Dzp1.0 mg/mL, 20μL; *i.p.*). After 30 min of the treatments, the animals were added to Petri dishes containing the same water of the aquarium, marked with four quadrants and the locomotor activity was analyzed by counting the number of line crossings, during 5 minutes. Animals that did not receive treatments (Naïve) were considered as baseline (100% of locomotor activity).

Test light & dark

The anxiolytic-like effect of F-EtOAc was investigated through the Light & Dark Test, based on the methodology proposed by Gebauer et al.[24] The animals (n=6/group) were treated intraperitoneally with F-EtOAc (1.0 or 2.5 or 5.0 mg/mL, 20μL; *i.p.*) or vehicle (Control, 3% DMSO, 20μL) or diazepam (Dzp; 1.0 mg/mL, 20μL; *i.p.*). An untreated group (Naïve) was included. After 30 min of the treatments, the animals were added to the light zone of the glass aquarium (30 x 15 x 20 cm), which was divided into light and dark zones, with drug-free water. The anxiolytic-like effect was characterized by the presence of the animals in the light zone, during 5 min of analysis.

Involvement of the serotonergic system

Groups of animals (n=6) received cyproheptadine ($5\text{-}HT_{2A}$ antagonist) or pizotifen ($5\text{-}HT_1$ antagonist and $5\text{-}HT_{2A}/_{2C}$) orally (*p.o.*), either at a dose of 0.8 mg/mL or granisetron ($5\text{-}HT_{3A}/_{3B}$ antagonist, 0.5 mg/mL, *p.o.*)30 minutes before F-EtOAc (1.0 mg/mL, *i.p.*) or fluoxetine (Flx; 1.25×10^{-3}mg/mL, *i.p.*). Subsequently, the Light & Dark Test was performed as cited previously.

Involvement of the GABAergic system

To evaluate the involvement of the GABAergic system, other groups of animals (n=6/each) received Flumazenil (0.1 mg/mL) 15 minutes before F-EtOAc (1.0 mg/mL) or Diazepam (1.0 mg/mL) and the Light & Dark Test was performed as previously described.

Alcohol withdrawal-induced anxiety

Alcohol withdrawal-induced anxiety was investigated in adult zebrafish, based on the method described by Ferreira et al.[25] Yellow sugar cane brandy (YSCB) was used as the source of ethanol (38% EtOH). Then the animals (n=6/group) were divided into seven groups:

a) Group I - Naïve (without treatments);

b) Group II - Vehicle - DMSO 3% (20μl, i.p.);

c) Group III - From the 1st to the 5th day: YSCB (20μL, p.o.).

d) Group IV - YSCB (20μL, p.o.), from the 1st to the 5th day. On day 11: F-EtOAc (1.0 mg/mL; i.p.);

e) Group V- YSCB (20μL, p.o.), from day 1 to day 5. On the 11th day: F-EtOAc (2.5 mg/mL; i.p.);

f) Group VI - YSCB (20μL; p.o.), from day 1 to day 5. On day 11: F-EtOAc (5.0 mg/mL; i.p.);

g) Group VII - YSCB (20μL; p.o.), from the 1st to the 5th day. On the 11th day: Diazepam (1.0 mg/mL; i.p.).

After 1 h of the oral treatments and 30 min of the intraperitoneal treatments, the animals were individually added to the light zone of the aquarium and the anxiolytic-like effect was characterized by the permanence of the animals in the light zone during 5 min of analysis. Reversion of anxiety caused by the abstinence crisis was evaluated only on the 11th day, where aZF's (n = 6/group) received F-EtOAc (1.0 or2.5 or 5.0 mg/mL; *i.p.*) or Diazepam (1.0 mg/mL; *i.p.*).

Statistical analysis

Results were expressed as mean±standard deviation of the mean for *in vitro* tests (n=3) as well as mean±standard error of the mean for *in vivo* tests (n=6/group). After confirming the distribution of normality and homogeneity of data, differences between the groups were submitted to analysis of variance (one-way ANOVA), followed by *Tukey* test, using the software GraphPad Prism 7.0. The level of statistical significance was considered at 5% ($p<0.05$).

Results

Toxicity

EtCNeem was shown to be toxic to *A. salina* nauplii (LC_{50}=288.46μg/mL). Through the extract saponification, it was observed that none of the obtained fractions caused the mortality of 50% of the *A. salina* nauplii (LC_{50} > 1.000μg/mL), in addition to not being toxic in adult zebrafih within 96 h of analysis (LD_{50}> 5.0 mg/mL).

Antioxidant activity

F-EtOAc of EtCNeem showed higher antioxidant potential against DPPH (EC_{50} = 21.6 ± 0.07μg/mL). Pearson's correlation coefficient (*r*) indicated that such antioxidant activity was correlated with the

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phenol ($r = 0.4135$) and flavonoid ($r = 0.9924$) contents in 41 % and 99 %, respectively. That is why this fraction was chosen for testing of anxiolytics.

Preliminary phytochemical screening and FT-IR analysis

The preliminary phytochemical analysis of the F-EtOAc disclosed the presence of phenolic, flavonoid, steroid and triterpenoid compounds. Through FT-IR, the presence of functional groups of these classes of chemical constituents was detected, as shown in Figure 1, Table 1.

Table 1 List of functional groups of chemical constituents present in Et-EtOAc the EtCNeem

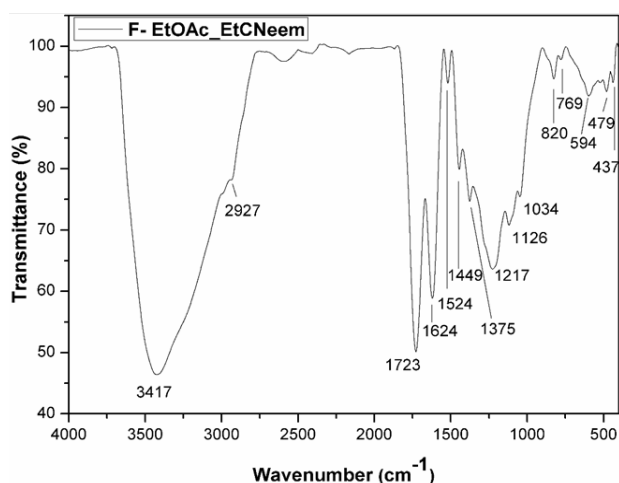| Functional group	Vibration (Stretch/Bend)	Range (cm-1)	F-EtOAc_ EtCNeem
Alkanes	C-H stretch	3000-2800	✔
	C-H bend	1500-1440	✔
Alkenes	C-H stretch	3200-3000	-
Aromatic rings	C=C stretch	1600-1400	✔
Hydroxyl	O-H stretch	3600-3200	✔
Carbonyl	C=O stretch	1610-1550	✔
Amino acid	C=O stretch	1660-1600	✔
Aldehydes	C=O stretch	1750-1625	✔
Amines	C-H stretch	2850-2700	-
Nitriles	C≡N stretch	2500-2000	✔
Amides	N-H stretch	3500-3100	-
	C=O stretch	1670-1600	✔
Ethers	C-O-C stretch	1260-1040	✔

Figure 1 FT-IR spectrum (400-4000 cm⁻¹) of EtOAc the EtCNeem.

Measurement of total phenols and flavonoids

F-EtOAc is rich in phenolic compounds (454.32±0.005 mg of

EGA/g of extract), as well as in flavonoids (41.12±0.045 mg EQuer/g of extract).

Locomotor activity evaluation (Open Field Test)

All doses of F-EtOAc (**$p<0.05$) and Diazepam (****$p<0.0001$) decreased the locomotor activity of adult zebrafish in comparison to the naive and vehicle groups ($F_{5,30} = 10.01$; Figure 2).
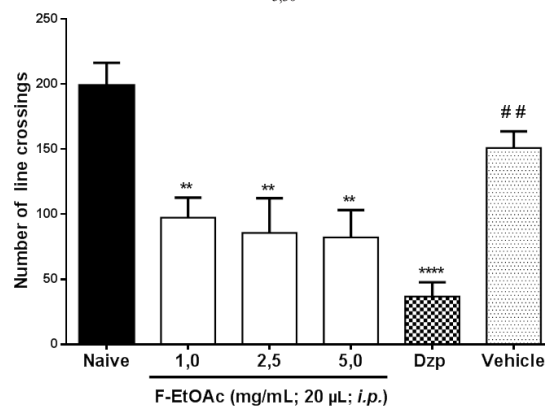

Figure 2 Effect of Et-EtOAc the EtCNeem under the locomotor behavior of zebrafish (*Danio rerio*) adult in the Open Field Test (0-5 min). F-EtOAc – Fraction ethyl acetate; Dzp – Diazepam (1.0 mg/mL, *i.p.*); Vehicle (DMSO 3%). Values represent the mean ± standard error of the mean for 6 animals/group; ANOVA followed by *Tukey* ****$p<0.0001$, **$p<0.01$ *vs* Naive; ##$p<0.01$ *vs* Dzp.

Preliminary anxiolytic activity (Light & Dark Test)

The F-EtOAc (1.0 or 2.5 or 5.0 mg/mL; *i.p.*) and Diazepam (1.0 mg/mL; *i.p.*) increased (****$p< 0.0001$ vs naive or vehicle) the permanence in the light zone of the Light & Dark Test ($F_{5, 30} = 60.4$; Figure 3).
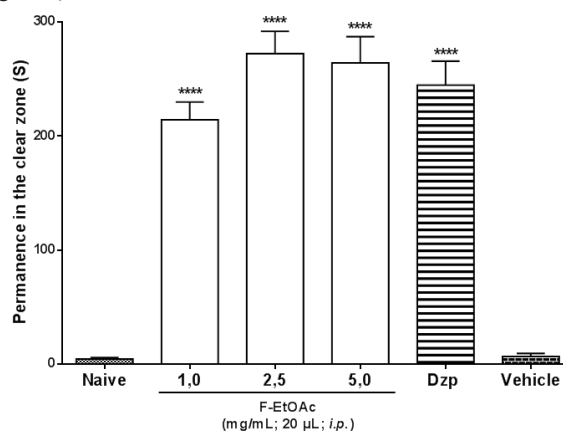

Figure 3 Effect of Et-EtOAc the EtCNeemon adult zebrafish in the Light & Dark Test (0-5 min). The numbers above each column indicate percentage of permanence in the light zone. Naïve–group without treatment; Vehicle–Control (DMSO 3%; 20 µl; *i.p.*); Dzp–Diazepam (1.0 mg/mL; *i.p.*). Values represent the mean ± standard error of the mean for 6 animals/group; ANOVA followed by *Tukey*. ****$p<0.0001$ *vs* naive and vehicle.

Involvement of the Serotonergic System (5-HT)

Involvement of the 5-HT$_{2A}$ System

Cyproheptadine not reduced the anxiolytic-like effect of F-EtOAc (1.0 mg/mL, *i.p.*). However, cyproheptadine (####$p<0.0001$) reduced the anxiolytic-like effect of fluoxetine (Flx; $1.25×10^{-3}$ mg/mL; 20µL; *i.p.*) ($F_{6,35} = 135.6$; Figure 4A).

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Involvement of the 5-HT$_1$ and 5-HT$_{2A/2C}$ systems

The Pizotifen reduced ($^{\#\#\#\#}p<0.0001$) the anxiolytic-like effect of F-EtOAc (1.0 mg/mL; *i.p.*) and fluoxetine (Flx; 1.25×10^{-3} mg/mL; 20μL; *i.p.*) (F$_{6,35}$=132.7; Figure 4B).

Involvement of the 5-HT$_{3A/3B}$ system

The Granisetron reduced ($^{\#\#\#\#}p<0.0001$) the anxiolytic-like effect of F-EtOAc (1.0 mg/mL; *i.p.*) and fluoxetine (1.25×10^{-3} mg/mL; 20μL; *i.p.*) (F$_{6,35}$= 137.7; Figure 4C).

Involvement of the GABAergic system

Flumazenil reduced ($^{\#\#\#\#}p<0.0001$) the anxiolytic-like effect of F-EtOAc (1.0 mg/mL; *i.p.*) and of Diazepam (1.0 mg/mL; *i.p.*) (F$_{6,35}$ = 68.49; Figure 5).
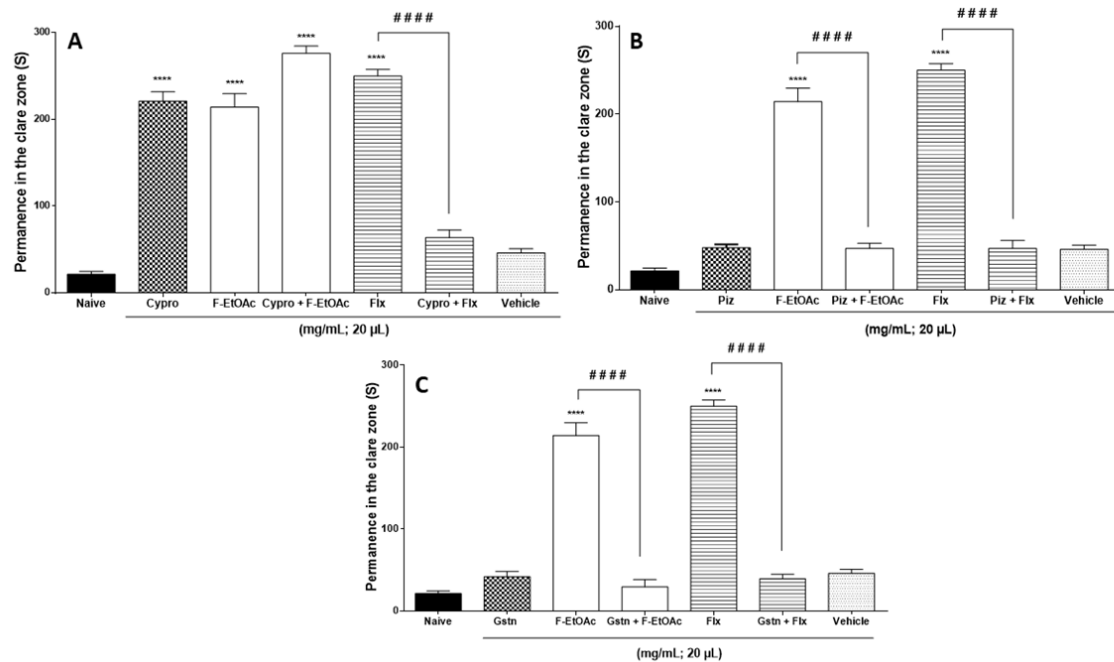

Figure 4 Anxiolytic-like effect of F-EtOAc after pretreatment with cyproheptadine (A), pizotifen (B), and granisetron (C) in adult zebrafish in the light/dark test (0–5 min). Naive, untreated fishes; Cypro, cyproheptadine (0.8 mg/mL; 20μL; *p.o.*); Piz, pizotifen (0.8 mg/mL;20μL; *p.o.*); Gstn, granisetron (0.5 mg/mL; 20μL; *v.o.*). F-EtOAc (1.0 mg/mL; 20μL; *i.p.*); Flx, Fluoxetine (1.25 × 10^{-3} mg/mL; 20μL; *i.p.*);Vehicle: DMSO 3% (20μL; *i.p.*).Values are presented as group mean±} S.E.M. Differences between the groups were determined by ANOVA followed by Tukey's *post hoc* test. Significant differences: ****$p< 0.0001$ *vs.* naive or vehicle; $^{\#\#\#\#}p< 0.001$ *vs.* Flx or F-EtOAc.
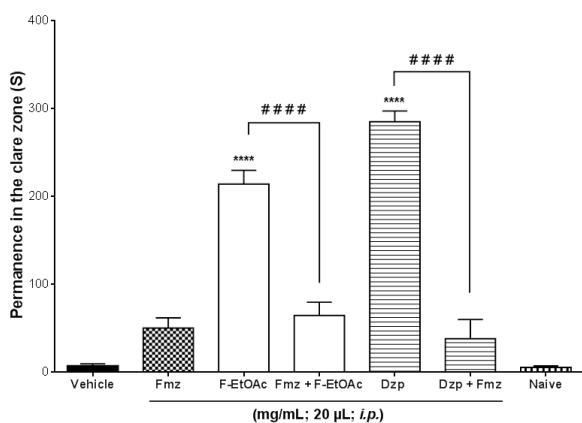

Figure 5 Effect of flumazenil under the anxiolytic-like action of F-EtOAc of EtCNeem in adult zebrafish (Danio rerio) on the Light & Dark Test (0-5min). Naïve–untreated animals; F-EtOAc (1,0 mg/mL,*i.p.*); Dzp–Diazepam (1,0 mg/mL; *i.p.*). Fmz–Flumazenil (0,1 mg/mL; *i.p.*). Vehicle–(3% DMSO 20µl, *i.p.*). Values represent the mean ± standard error of the mean for 6 animals/group. ANOVA followed by Tukey (****$p<0.0001$ *vs.* naive or vehicle; $^{\#\#\#\#}p<0.0001$ *vs.* Flzor F-EtOAc).

Alcohol withdrawal-induced anxiety

YSCB (38%; 20μL; *p.o.*) caused an anxiolytic-like effect from the 4th to the 5th day (****$p<0.0001$ *vs.* naïve or vehicle; F$_{6,385}$=58.63) of treatment. This effect was also observed during three days of abstinence: on the 6th and 7th day (****$p<0.0001$); on the 8th day (**$p<0.01$) (Figure 6). F-EtOAc (5.0 mg/mL, *i.p.*) significantly prevented (****$p<0.0001$) alcohol-withdrawal induced anxiety in adult zebrafish on the 11th day, significantly similar to the effect of Diazepam (Dzp; 1.0 mg/mL; *i.p.*; ****$p<0.0001$; F$_{6,35}$ = 16.86), Figure 7.

Discussion

Although the anxiolytic activity of *A. indica* leaf extracts has already been investigated in rodents,[16] the present study is the first to report the ability of *A. indica* bark ethanolic extract to reverse acute and chronic anxiety in zebrafish adult. The absence of toxicity after the saponification of the Neem bark ethanolic extract indicates its use in pharmacological anxiety tests, since the fractions obtained after the saponification were not toxic for *A. salina* and adult zebrafish. This absence of Neem toxicity was also demonstrated in the study by Kanagasanthosh et al.[26]

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The phenolic and flavonoid compounds present in ethanolic extracts of medicinal plants constitute classes of secondary metabolites responsible for the antioxidant action against DPPH radicals.[27] Our results showed F-EtOAc antioxidant action against the DPPH radical, being the most promising in relation to the presence of phenolic compounds (41.35%) and flavonoids (99.24%).

The antioxidant action of phenolic compounds from medicinal plants has been correlated with antidepressant and anxiolytic potentials.[28] In these pathological states, there are higher levels of free radical production in the brain, which results in depletion of the tripeptide glutathione (GSH), a redox regulator, which participates in the maintenance of oxidative homeostasis and reactive oxygen species (ROS) detoxification in brain cells.[28] The study by Jaiswal et al.,[16] indicated that low doses of *A. indica* leaf extract produced anxiolytic effects in rodents. In our study, it was possible to detect the anxiolytic-like effect on adult zebrafish of the F-EtOAc (1.0 or 2.5 or 5.0mg/mL) obtained from the Neem bark (Figure 3).

The analysis of the locomotor activity can be used as a hyperactivity evaluation parameter, indicating anxiety. The benzodiazepines used in the treatment of anxiety decrease the locomotor activity of adult zebrafish.[2,25,29] The F-EtOAc of EtCNeem had anxiolytic effect on the adult zebrafish, as it reduced the animals' locomotor activity (Figure 2).[30] In order to confirm anxiolytic behavior of F-EtOAc of EtCNeem, the Light & Dark test in an aquarium was performed, one of the methods most frequently used in anxiolytic tests with adult zebrafish (*Danio rerio*). All doses of EtOAc increased the animals' permanence in the clear area of the aquarium, indicating anxiolytic effect (Figure3).

Serotonin (5-hydroxytryptamine; 5-HT) is a monoamine neurotransmitter, which modulates mood, emotion and defensive, social and anxiety behaviors.[31] The genes encoding serotonin receptors in zebrafish show high homology with the corresponding human genes.[32] The anxiolytic potential of F-EtOAc was assessed after pretreatment with cyproheptadine antagonists (a 5-HTR_{2A} antagonist), pizotifen (a 5-HTR_1 and $5\text{-HTR}_{2A/2C}$ antagonist) and granisetron (an antagonist of 5-HTR_3). Fluoxetine was used as a positive control. Figure 4A shows that cyproheptadine did not reverse the anxiolytic effect of F-EtOAc, as the treated animals continued the clear region of the aquarium (****$p<0.001$ *vs.* naive/vehicle). Nevertheless, the anxiolytic activity of F-EtOAc was successfully inhibited by pretreatment with pizotifen (Figure 4B) and granisetron (Figure 4C), which reduced the animals' permanence in the clear zone to basal levels ($p<0.0001$ *vs.* F-EtOAc) respectively. These results suggest that the mechanism of action of F-EtOAc involves $5\text{-HTR}_1/5\text{-HTR}_{2C}$ and 5-HTR_3, but not 5-HTR_{2A}. Additionally, all antagonists have abolished the effects of fluoxetine (Figure 4 A, B and C).

Flumazenil is a $GABA_A$ receptor antagonist with anxiogenic properties,[33] responsible for antagonizing the effects of benzodiazepines, such as diazepam, including anxiolytic, sedative and hypnotic effects. In the literature, many researchers have used flumazenil as a tool to identify possible endogenous and exogenous ligands in benzodiazepine receptors in rodents,[34] as well as in adult zebrafish.[2] In this study, the reversion of anxiolysis through pretreatment with flumazenil suggests that the anxiolytic-like effect of F-EtOAC of EtCNeem depends on the $GABAR_A$ (Figure 5).

Studies have shown that acute exposure to alcohol acts as a potent anxiolytic, because when it enters the bloodstream, it penetrates the brain and stimulates neurons to release extra serotonin and dopamine,

neurotransmitters that regulate pleasure, mood, and anxiety.[35] Moreover, alcohol can affect neurotransmitters, such as by inhibiting the glutamatergic system and stimulating the GABAergic,[36] initially resulting in a calming effect.[37]

Sena et al.,[38] used distilled cachaça as a source of ethanol for the induction of anxiety in rodents. Benneh et al.,[2] used ethanol withdrawal (0.5%; v/v) to induce chronic anxiety in zebrafish by ethanol withdrawal, and then used the *Maerua angolensis* trunk bark extract to treat anxiety. In our study, yellow sugar cane brandy induced chronic anxiety in zebrafish after withdrawal on day 6, and abstinence was verified from day 8 to 10 without treatment with brandy (Figure 6). F-EtOAc (5.0mg/mL; *i.p.*) was shown to be effective in reversing anxiogenesis in adult zebrafish (Figure 7).
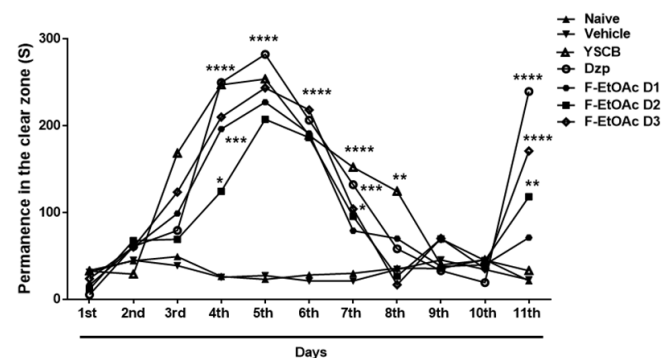

Figure 6 Effect of F-EtOAc on anxiety in adult zebrafish, induced by alcohol withdrawal, in the light and dark test (0-5min). Group I - Naive - untreated animals (Control). Group II - Vehicle (DMSO 3%; 20μl, *i.p.*; 1st-11th day). Group III - YSCB - yellow sugar cane brandy (EtOH 38%, 20μL; *p.o.*; 1st-5th day). Group IV - F-EtOAc D1 (1,0 mg/mL; *i.p.*; 11th day). Group V - F-EtOAc D2 (2,5 mg/mL; *i.p.*; 11th day). Group VI - EtOAc D3 (5,0 mg/mL; *i.p.*, 11th day). Group VII - Dzp-Diazepam (1.0 mg/mL, *i.p.*; 11th day).
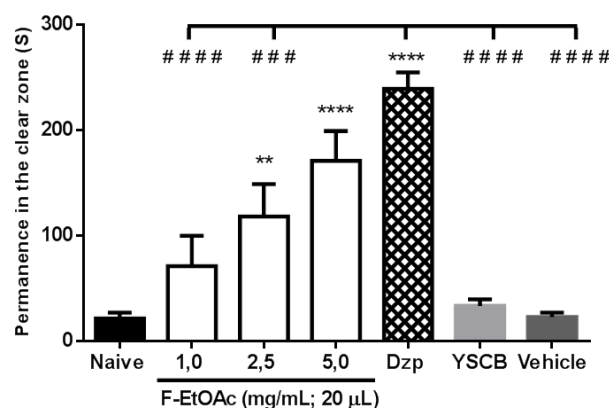

Figure 7 Prevention of anxiety induced by alcohol withdrawal (11th day) by F-EtOAc in the Light & Dark test (0–5 min). The values represent the mean ± standard error of the mean (E.P.M.) for 6 animals/group. Naive - untreated animals. YSCB - yellow cane spirit (38% EtOH); Dzp - Diazepam (1.0 mg/mL; *i.p.*). Vehicle - 3% DMSO (20μL; *i.p.*). ANOVA followed by *Tukey* (**** $p<0.0001$, **$p<0.01$ *vs.* Naïve or YSCB or Vehicle; ####$p<0.0001$ *vs.* Dzp).

Conclusion

The present study showed the pharmacological potential of the ethanolic extract of Neem bark. Our findings demonstrated that the F-EtOAc, obtained after saponification of EtCNeem, showed to be

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rich in phenolic and flavonoid compounds with antioxidant potential, as well as a nontoxic, sedative and anxiolytic-like effect on adult zebrafish via the serotoninergic (5-HT$_1$, 5-HT$_{2A/2C}$ and 5-HT$_{3A/3B}$), GABAergic systems and anxiolytic effect in the chronic anxiety test. Thus, this study adds new evidence and highlights the potential of *A. indica* A. Juss for the development of plant-based remedies with anxiolytic properties.

Acknowledgments

Conflicts of interest

The authors declare no conflicts of interest.

References

1. *Depression and other common mental disorders: global health estimates*. World Health Organization; 2017. 1–24 p.

2. Benneh CK, Biney RP, Mante PK, et al. Maerua angolensis stem bark extract reverses anxiety and related behaviours in zebrafish-Involvement of GABAergic and 5-HT systems. *Journal of Ethnopharmacology*. 2017;207:129–145.

3. Kato H, Tsuji M, Miyagawa K, et al. Repeated exposure to stress stimuli during ethanol consumption prolongs withdrawal-induced emotional abnormality in mice. *European Journal of Pharmacology*. 2013;721(1-3):29–34.

4. Carmichael O, Lockhart S. The Role of Diffusion Tensor Imaging in the Study of Cognitive Aging. *Curr Top Behav Neurosci*. 2012;11:289–320.

5. Hood SD, Norman A, Hince DA, et al. Benzodiazepine dependence and its treatment with low dose flumazenil. *British Journal of Clinical Pharmacology*. 2014;77(2):285–294.

6. Graf H, Walter M, Metzger CD, et al. Antidepressant-related sexual dysfunction-Perspectives from neuroimaging. *Pharmacology Biochemistry and Behavior*. 2014;121:138–145.

7. López-Rubalcava C, Estrada-Camarena E. Mexican medicinal plants with anxiolytic or antidepressant activity: Focus on preclinical research. *Journal of Ethnopharmacology*. 2016;186:377–391.

8. Harro J. Animals, anxiety, and anxiety disorders: How to measure anxiety in rodents and why. *Behavioural Brain Research*. 2018;352:81–93.

9. Frantz S. Therapeutic area influences drug development costs. *Nature reviews Drug Discovery*. 2004;3(6):466–467.

10. Panula P, Sallinen V, Sundvik M, et al. Modulatory Neurotransmitter Systems and Behavior: Towards Zebrafish Models of Neurodegenerative Diseases. *Zebrafish*. 2006;3(2):235–247.

11. Venuprasad MP, Kumar Kandikattu H, Razack S, et al. Phytochemical analysis of Ocimum gratissimum by LC-ESI-MS/MS and its antioxidant and anxiolytic effects. *South African Journal of Botany*. 2014;92:151–158.

12. Fernandez SP, Nguyen M, Yow TT, et al. The flavonoid glycosides, myricitrin, gossypin and naringin exert anxiolytic action in mice. *Neurochemical Research*. 2009;34(10):1867–1875.

13. Batista FLA, Luiza MG, Araújo JIB, et al 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). *Biomedicine & Pharmacotherapy*. 2018;108:408–416.

14. Khosla P, Singh BSJ, Srivastava RK. Antinociceptive Activity of Azadirachta Indica Antinociceptive Activity of Azadirachta Indica (Neem) in Rats. *Indian Journal of Pharmacology*. 2000;32(6):372–374.

15. Maiti R, Kumar S, Acharya S, e al. Role of aqueous extract of Azadirachta indica leaves in an experimental model of Alzheimer′s disease in rats. *International Journal of Applied and Basic Medical Research*. 2013;3(1):37–47.

16. Acharya SB, Jaiswal AK, Bhattacharya SK. Anxiolytic activity of Anzadirachta indica leaf extract in rats. *Indian Journal of Experimental Biology*. 1994;32(7):489–491.

17. Matos FJA. Introduction to Experimental Phytochemical, 3rd ed; 2009.

18. Scroder L. Obtencao De Acidos Graxos De Biomassa Umida De Microalgas Atraves De Saponificaco. Federal University of Paraná; 2013.

19. Morais SM, Lima KSB, Siqueira SMC, et al. Correlação entre as atividades antiradical, antiacetilcolinesterase e teor de fenóis totais de extratos de plantas medicinais de farmácias vivas. *Revista Brasileira de Plantas Medicinais*. 2013;15(4):575–582.

20. Slinkard K, Singleton VL. Total Phenol Analysis: Automation and Comparison with Manual Methods. Am J Enol Vitic. 1997;28:49–55.

21. Kosalec I, Bakmaz M, Pepeljnjak S, et al. Quantitative analysis of the flavonoids in raw propolis from northern Croatia. *Acta Pharmaceutica*. 2004;54(1):65–72.

22. Ekambaram SP, Perumal SS, Pavadai S. Anti-inflammatory effect of Naravelia zeylanica DC via suppression of inflammatory mediators in carrageenan-induced abdominal oedema in zebrafish model. *Inflammopharmacology*. 2017;25(1):147–158.

23. Magalhães FEA, de Sousa CÁPB, Santos SAAR, et al. Adult Zebrafish (Danio rerio): An Alternative Behavioral Model of Formalin-Induced Nociception. *Zebrafish*. 2017;14(5):422-429.

24. Gebauer DL, Pagnussat N, Piato AL, et al. Effects of anxiolytics in zebrafish: Similarities and differences between benzodiazepines, buspirone and ethanol. *Pharmacology Biochemistry and Behavior*. 2011;99(3):480–486.

25. Ferreira MKA, da Silva AW, Silva FCO, et al. Anxiolytic-like effect of chalcone N-{(4'-[(E)-3-(4-fluorophenyl)-1-(phenyl) prop-2-en-1-one]} acetamide on adult zebrafish (Danio rerio): involvement of the GABAergic system. *Behavioural Brain Research*. 2019;374:111871.

26. Kanagasanthosh K, Shanmugapriyan S, Kavirajan V. Evaluation of Acute Toxicity, Anti-Inflammatory Activity and Phytochemical Screening of Ethanolic Extract of Azadirachta Indica Leaves. *Int J Res Dev Pharm L Sci*. 2015;4(5):1737–1742.

27. Magalhães FEA, Batista FLA, Serpa OF, et al. Orofacial antinociceptive effect of Mimosa tenuiflora (Willd.) Poiret. *Biomedicine and Pharmacotherapy*. 2018;97:1575–1585.

28. Afsar T, Razak S, Khan MR, et al. Anti-depressant and anxiolytic potential of Acacia hydaspica R. Parker aerial parts extract: Modulation of brain antioxidant enzyme status. *BMC Complementary and Alternative Medicine*. 2017;17(1):1–12.

29. Gupta P, Khobragade S, Rajaram S, et al. Assessment of locomotion behavior in adult Zebrafish after acute exposure to different pharmacological reference compounds. *Drug Development and Therapeutics*. 2014;5(2):127–133.

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30. Siebel AM, Bonan CD, Silva RS da. Zebrafish como modelo para estudos comportamentais. In: *Biotecnologia aplicada à saúde: Fundamentos e aplicações*; 2015. 17–55 p.

31. Maximino C, Herculano AM. A review of monoaminergic neuropsychopharmacology in zebrafish. *Zebrafish*. 2010;7(4):359–378.

32. Klee EW, Schneider H, Clark KJ, et al. Zebrafish: A model for the study of addiction genetics. *Human Genetics*. 2012;131(6):977–1008.

33. Bernik M, Ramos RT, Hetem LAB, et al. Effect of single doses of pindolol and d-fenfluramine on flumazenil-induced anxiety in panic disorder patients. *Behav Brain Res*. 2019;357-358:82–87.

34. Viswanatha GL, Venkataranganna MV, Prasad NBL. Ameliorative potential of Colebrookea oppositifolia methanolic root extract against experimental models of epilepsy: Possible role of GABA mediated mechanism. *Biomedicine and Pharmacotherapy*. 2017;90:455–465.

35. Banerjee N. Neurotransmitters in alcoholism: A review of neurobiological and genetic studies. *Indian Journal of Human Genetics*. 2014;20(1):20-31.

36. Zaleski M, Morato GS, Silva VA Da, et al. Neuropharmacological aspects of chronic alcohol use and withdrawal syndrome. *Revista Brasileira de Enfermagem*. 2004;26(Suppl 1):S40–S42.

37. Araujo-Silva H, Pinheiro-da-Silva J, Silva PF, et al. Individual differences in response to alcohol exposure in zebrafish (Danio rerio). *Plos One*. 2018;13(6):e0198856.

38. Sena MCP, Nunes FC, Salvadori MGSS, et al. Chronic consumption of distilled sugarcane spirit induces anxiolytic-like effects in mice. *Clinics*. 2011;66(5):873–878.