

Original Research

Hippocampus reidi extract, a marine natural product, attenuates NF- κ B expression and suppresses inflammatory activity *in vitro* and *in vivo*

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Summary. Seahorses (*Hippocampus reidi*) are used in Brazilian folk medicine to treat asthma and other inflammatory conditions. In this study, we explored the chemical profile as well as the anti-inflammatory potential of *H. reidi* hydroalcoholic extract *in vitro* and *in vivo*. First, we explored the chemical profile of this extract using high performance liquid chromatography and high-resolution mass spectrometry, a combinatorial analysis that demonstrated that the *H. reidi* is rich in peptides. Then, the cytotoxic activity of *H. reidi* on spleen cells, its modulatory effect on cytokines and nitric oxide production and its stabilizing capacity of red blood cell membranes was evaluated *in vitro*. Finally, we evaluated NF- κ B gene expression. *In vivo* studies were performed using a peritonitis mouse model. Leukocyte recruitment to the peritoneum was evaluated, and IL-6 was measured by ELISA. The *H. reidi* extract attenuated the production of Th2 and Th1 cytokines and modulated the immunoregulatory cytokine. In addition, we observed a reduction in nitric oxide production as well as a stabilization of the erythrocyte membrane in addition to an inhibition of transcriptional factor NF- κ B. The *H. reidi* extract showed an anti-inflammatory effect *in vivo* by reducing the total leukocyte and neutrophil migration and IL-6 concentration in the peritoneal cavity. Thus, our study revealed that *H. reidi* hydroalcoholic extract possesses anti-inflammatory activity *in vitro* and *in vivo* and may be a candidate for the isolation of molecules for further study and that *H. reidi* hydroalcoholic extract might also represent a component of a therapeutic arsenal of inflammatory disorders.

Industrial Relevance. *H. reidi* has been commonly used in folk medicine for the treatment of inflammatory disorders. This study is the first to demonstrate the anti-inflammatory activity of *Hippocampus reidi* *in vitro* and *in vivo*. Additional work is needed to confirm which molecules are responsible for the observed effects and better understand the mechanisms whereby such effects occur.

Keywords. Natural Products; *Hippocampus reidi*; Anti-inflammatory; Peptides.

INTRODUCTION

Inflammation is linked to many widespread diseases including rheumatoid arthritis, chronic asthma, multiple sclerosis, gastritis, type I diabetes, psoriasis and cancer. Many of these diseases are debilitating, impacting quality of life and increasingly becoming a global public health problem ¹.

Pharmacological strategies such as glucocorticoid and nonsteroidal anti-inflammatory drugs (NSAIDs) are used to treat inflammatory disorders ¹. However, although anti-inflammatory drugs are effective for the temporary relief of symptoms, drug-induced severe side effects, including gastrointestinal and renal damage, have been observed ². Therefore, new research has been conducted in an effort to find new drugs with lower costs that are associated with lower incidence of side effects. Thus, marine organisms have been experimentally tested to represent an alternative to currently available anti-inflammatory drugs ^{3, 4, 5}. *Hippocampus reidi* Leach belongs to *Syngnathidae* of *Syngnathiformes* in *Steichthyes* is a fish of vertebrate phylum. Animals in *Syngnathidae* can be used as traditional Chinese medicine material ⁶. Recent pharmacological studies suggested that Chinese seahorse (*Hippocampus kuda* Bleeler) revealed that had variously bioactivities such as anti-tumor, anti-aging, anti-inflammatory, anti-asthmatic, antioxidant, anti-fatigue activities, platelet aggregation inhibition; N-methyl-N'-nitro-N-nitrosoguanidine-induced rat gastric carcinogenesis inhibition; Ca²⁺ channel blocking and invigorant for the treatment of erectile dysfunction (ED). ⁶⁻⁹. However, no previous work has explored the immune modulatory activity of another *Hippocampus* species, *Hippocampus reidi* (seahorse), the most abundant in Brazil ¹⁰ (fig.01).

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Figure 1. *Hippocampus reidi*

As part of an effort to discover new bioactive natural products from marine resources, we have identified the marine species *Hippocampus reidi* as one of the most cited natural products used for the treatment of asthma and other inflammatory conditions, in an ethnobotanical survey in the City of São Francisco do Conde (SFC), Salvador, Bahia, Brazil (unpublished data). Thus, the purpose of the present study was to investigate the chemical profile and the cytotoxicity as well as the anti-inflammatory potential of the hydroalcoholic extract of *H. reidi* *in vitro* and *in vivo*.

MATERIALS AND METHODS

Animals. Male BALB/c mice at 4 to 6 weeks of age and Wistar rats at 2 to 3 months old were obtained from the animal facilities of the Fundação Oswaldo Cruz (FIOCRUZ), Bahia, Brazil and used for subsequent experiments. The animals were maintained with free access to food and water.

Ethical approval. All experimental procedures were approved by the Ethical Committee for Animals Use of the Health Science Institute (CEU-ICS), Federal University of Bahia, Brazil (protocol number: 028/2012) and conducted according to international standards.

Preparation of *H. reidi* hydroalcoholic extract. Live adult seahorses were collected from a watercourse of Santa Cruz, in the city of Itamaracá, Pernambuco, Brazil (Authorization and license (SISBIO), nº 39294-1). These organisms were identified as *Hippocampus reidi* in Project Hippocampus from the Marine Aquaculture Laboratory, according to Silveira et al (2014)¹¹. To prepare the hydroalcoholic extract of *H. reidi*, the seahorse samples were dried and extracted by maceration using ethanol and water at a 1:1 ratio for 16 h at room temperature. After filtration, the extracts were concentrated at 50° C. Subsequently, the material was lyophilized at -50° C.

Sample preparation and analysis. The *Hippocampus heidi* extract (515.7 mg) was dissolved in methanol and extracted using solid phase extraction (SPE). The cartridge (Waters) was sequentially conditioned with methanol and water, and the cartridge was not dried. The extract was passed through the cartridge and eluted with water and methanol. The eluates were dried under reduced pressure in a rotary evaporator at 40°C to yield 327.6 mg of an aqueous fraction and 110.0 mg of a methanol fraction. After evaporating to dryness by a rotary evaporator, the residues were dissolved in methanol and water, filtered through a 0.22-µm nylon syringe filter (Whatman) and injected into the UPLC/Xevo G2XS Q-TOF MS. The standard stocks and working solutions were stored at 4 °C. All solvents (water, acetonitrile and formic acid) were LC-MS grade or equivalent reagents purchased from Sigma-Aldrich (Dorset, UK). The infrared absorption spectra were recorded in KBr pellets using a Varian 640 FT-IR spectrophotometer equipped with a PIKE ATR accessory operating in the 4000-400 cm⁻¹ range. TLC plates were run using 60 F254 silica gel (Macherey-Nagel). The thin layer chromatographic profiles were realized with fractions obtained by SPE extraction of *H. Heidi*. Chromatoplates were realized using methanol as the mobile phase. A specific ninhydrin reagent spray was used to identify free amino groups among the constituent amino acids.

UPLC/ XEVO-G2XSQTOF analysis of peptides. The XEVO-G2XSQTOF mass spectrometer (Waters, Manchester, UK) was connected to an ACQUITY UPLC system (Waters, Milford, MA, USA) via an electrospray ionization (ESI) interface. The chromatographic separation of peptides was performed on an ACQUITY UPLC system with a conditioned autosampler at 4°C using an Acquity BEH C18 column (50 mm × 2.1 mm i.d., 1.7 µm particle size) (Waters, Milford, MA, USA). The column temperature was maintained at 40 °C. The mobile phase, comprising 0.1% formic acid in water (solvent A) and acetonitrile (solvent B), was pumped at a flow rate of 0.4 mL min⁻¹. The following gradient elution programme was used: 0-5 min with 10-50% B and 0.5-10 min with 50-95% B. The injection volume was 5 µL. The XEVO-G2XSQTOF mass spectrometer was used in a positive ESI mode, and the scan range was from 50 to 1200 *m/z* for data acquisition using UPLC/MS^E, enabling the acquisition of both precursor and product ion data in one injection. The following source conditions were used: capillary voltage, 2.0 kV; sample cone, source temperature, 100 °C; desolvation temperature, 250 °C; cone gas flow rate, 20 L h⁻¹; and desolvation gas (N₂) flow rate, 600 L h⁻¹. All analyses were performed using lockspray to ensure accuracy and reproducibility. Leucine-enkephalin (5 ng mL⁻¹) was used as a standard or reference compound to calibrate mass spectrometers during analysis and introduced by a lockspray at 10 µL min⁻¹ for accurate mass acquisition. The acquisition and analysis of data were controlled using Waters MassLynx v 4.1 software. The BioLynx software package was used to identification of peptides.

Cytotoxicity assay. The cytotoxicity of *H. reidi* was evaluated as described by Stevigny et al (2002)¹² using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma), colorimetric method based on the cleavage of the reagent by dehydrogenases in viable cells¹³. Stock solutions of the hydroalcoholic *H. reidi* extract were prepared at 10

mg/mL in DMSO. The solutions were further diluted to final concentrations of 2000-7.8 µg/mL. The spleen cell cultures were incubated for 72 hours at 37°C in an atmosphere with 5% CO₂. All experiments were performed at least in duplicate.

Cytokine production in spleen cells culture. Mouse spleen cells were aseptically obtained and washed twice in RPMI medium by centrifugation at 252 g for 10 min. The pellet was resuspended in RPMI medium supplemented with 200 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, 5-Mercaptoethanol and 10% FCS (Gibco, Paisley, UK). The concentration of spleen cells was adjusted to 5x10⁶ viable cells/well using trypan blue in a haemocytometer. Six wells for each concentration of *H. reidi* (125, 62, and 31 µg/mL) were plated in 96-well flat-bottomed tissue culture plates (Costar, Cambridge, MA, USA) in a final volume of 200 µL per well. Pokeweed (PWM) (Sigma, St Louis, MA, USA) at 5 µg/mL was added in the presence or absence of *H. reidi* extract. After 72 hours of culture at 37 °C in an atmosphere of 5% CO₂, the supernatants were collected and IL-4, IL-5, INF-γ and IL-10 levels were determined by ELISA according to the manufacturer's instructions (BD Pharmingen, USA). Cell proliferation was estimated based on the MTT-tetrazolium method.

Effect of *H. reidi* extract on nitric oxide (NO) release from mouse macrophages. The estimation of the NO levels in the supernatant from *H. reidi* treated/untreated cells was performed as previously described by Chandrasekaran, 2010¹⁴. Briefly, peritoneal macrophages were pre-incubated with 125, 62 or 31 µg/mL of *H. reidi* extract for 1 h and were then incubated with LPS (5 µg/mL) for 24 h. After incubation, the supernatants were collected and analysed for nitrite based on Griess' reaction¹⁵. Briefly, the culture supernatant was mixed with Griess' reagent and incubated at room temperature for 5 min. The absorbance was measured at 540 nm using a microplate reader. The amount of nitrite in the sample was determined using a sodium nitrite standard curve.

In vivo evaluation of anti-inflammatory activity. Peritonitis induced by lipopolysaccharide. In order to evaluate the in vivo anti-inflammatory effect of *H. reidi* extract mice were pre-treated with vehicle saline, v.o. (group I), *H. reidi* extract (100 mg/Kg, v.o. (group III) or s.c. (group IV) or dexamethasone (0.3 mg/Kg, v.o. (group V). After 1 h, LPS (1 µg/mL) of *Escherichia coli* dissolved in sterile saline solution was administered intraperitoneally (II group). Six hours after LPS administration leukocytes were harvested by washing the peritoneal cavities with 3 mL of saline solution containing EDTA. Aliquots of the washes were used to determine total cell counts in Neubauer chamber after dilution in Trypan blue solution. Differential leukocyte counts were performed using May-Grunwald-Giemsa-stained cytospin preparations. A neutrophil differential count of at least cells/mL was made in a blind fashion in accordance with standard morphological criteria¹⁶.

Cytokine quantification in the peritoneal lavage. The levels of IL-6 (pg/mL) were determined in accordance with the manufacturer's instructions (BD Pharmingen, USA).

Membrane stabilizing test. The anti-haemolytic effect of *H. reidi* extract on erythrocytes induced by heat was evaluated according to Shinde et al (1999)¹⁷ and Sakat et al (2010)¹⁸, with some modifications. Whole blood was obtained from mice and transferred to heparinized tubes. The blood was washed three times with an isotonic buffered solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4) for 10 minutes at 1000 g. The volume of blood was measured and reconstituted as 10% v/v suspension with normal saline. The reaction mixture (5 mL) comprised 4.5 mL of solutions with different concentrations of *H. reidi* extract (125, 62, or 31 µg/mL) and 500 µL of a 10% erythrocyte suspension. Aspirin (AAS) was used as a control treatment at 200 µg/mL. All tubes containing the mixture were incubated in a water bath at 56°C for 30 min. At the end of the incubation, the tubes were cooled under running tap water. The mixture was then centrifuged at 800 g for 5 min, and the absorbance of the supernatants was measured at 560 nm using an EZread 400 spectrophotometer (Biochrom, UK). The experiment was performed in triplicate for all concentrations tested. The percentage of inhibition of haemolysis was calculated using the following equation.

$$\% \text{ Inhibition of haemolysis} = 100 - (\text{AbsC} / \text{AbsH}) \times 100$$

Where:

AbsB = Absorption of blood at 560 nm

AbsH = Absorption of tested *H. reidi* concentration at 560 nm

NF-κB expression by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. RNA was isolated from mouse spleen cells using the RNeasy Mini Kit (Qiagen, Hamburg, Germany) according to the manufacturer's instructions. Subsequently, 0.3 µg of total RNA from each sample was reverse transcribed into cDNA using 200 U of Superscript III Reverse Transcriptase (Life Technologies) and 500 ng of Oligo(dT) (Life Technologies) according to the manufacturer's instructions. Sterilized and filtered DEPC-treated water was used in all cDNA reactions.

The PCR primers for the detection of NF-κB and β-actin were designed based on the sequences reported in GenBank using Primer Express software (Applied Biosystems). The selected parameters lacked or had low secondary structures or primer-primer interactions and high specificity validated by Blast in NCBI (see descriptions in Table 01). Both primers were designed on different exons to eliminate potential genomic DNA contamination. The cDNA samples derived from the investigated genes were detected using a QuantStudio 12K Sequence Detection System (Applied Biosystems) according manufacturer's instructions. Each RT-PCR was run with 10 ng of cDNA in a reaction containing 10 µL of 2X SYBR-PCR Master Mix (Applied Biosystems), 1 µL of the respective primer mix (NF-κB: Forward 100 nM and Reverse 100 nM; β-Actin: Forward 500 nM; Reverse 250 nM), and purified and deionized H₂O up to 20 µL. Relative quantification was performed using the comparative threshold cycle (ΔΔCT) method, as previously described¹⁹.

Before using the ΔΔCT method for quantification, a validation experiment was performed to verify that the efficiencies of target and control were approximately equal. The amplification of all samples was of the same efficiency for the precise quantification of real-time-PCR (RT-PCR) data. Serial five-fold dilutions, starting with 100 ng of cDNA from the control group, were used. The mean C_T values, measured in duplicate, versus the log₁₀ of the dilution were plotted. The values obtained from the linear regressions applied to these plots were also presented (not shown). The amplification efficiencies (E = 10^(-1/slope)) were close to 1.0 (100%).

Table 1. Description of primer design. The table shows the primer sequences, amplicon lengths and annealing temperatures.

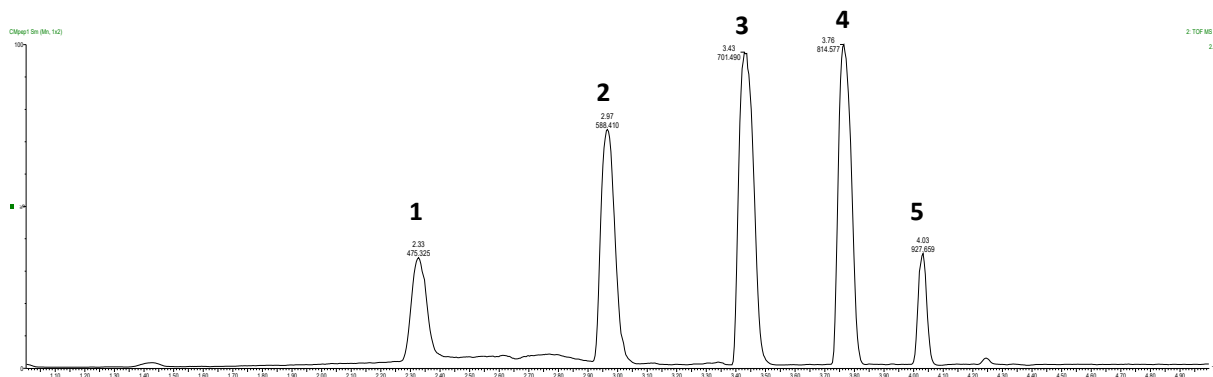
Gene	Sequences	Amplicon Length	T °C
B-actin Forward	ACCACACCTTCTACAATGAG	20	60°C
B-actin Reverse	ATCTGGGTCATCTTTTCACG	20	60°C
NFkB Forward	ATTCCGCTATGTGTGAAGG	21	60°C
NFkB Reverse	GTGACCAACTGAACGATAACC	21	60°C

Statistical analysis. One-way analysis of variance (ANOVA) and Tukey's post-test (for data with normal distribution) were used to determine the significance between experimental groups. Differences with p values ≤ 0.05 were considered statistically significant. Each experiment was repeated at least two times.

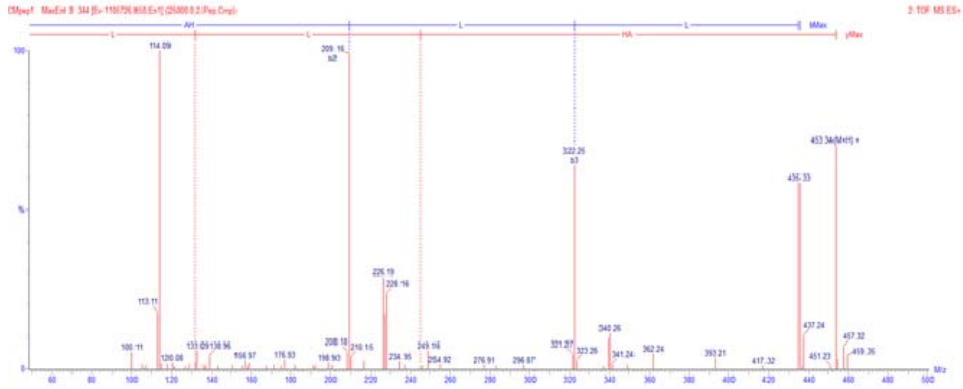
RESULTS

***H. reidi* hydroalcoholic extract is rich in peptides.** Figure 2 shows the chromatographic profile of the chromatoplate SPE fraction, revealing a predominant rose wine colouration (positive ninhydrin reaction) to the peptides. Analysis of the FTIR spectrum showed typical absorption bands corresponding to N-H stretching, suggesting that the substance contained peptides in the structure. The presence of an absorption band at 1635 cm^{-1} indicates the presence of an amide functional group. A band at 2930 cm^{-1} may indicate a carboxyl group. The presence of carboxyl and amino groups indicates the existence of amino acids, suggesting a polypeptide structure.

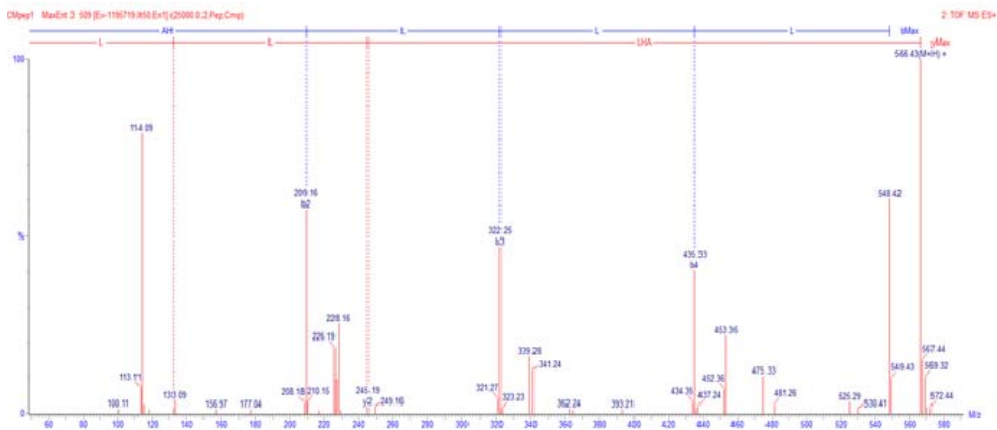
In this study, we isolated a mixture of five active peptides from seahorse from an SPE aqueous extract. The sequences presented amino acids histidine, alanine and leucine (or isoleucine) only. Because the two amino acids isoleucine and leucine have the same molecular weight was not possible to identify which of the two is part of the sequence. The Figure 3 presents the Base peak ion chromatogram of peptide mixture. The Figure 4 (A-E) present the molecular mass and amino acid sequence to the peaks 1 to 5. The peaks 1 m/z 453.34 [M+H]⁺, 2 m/z 566.43 [M+H]⁺, 3 m/z 679.51 [M+H]⁺, 4 m/z 792.59 [M+H]⁺ and 5 m/z 905.68 [M+H]⁺ corresponds to the sequences AHLL, AHLLL, AHLLLL, AHLLLLL and AHLLLLL, respectively. The amino acid Leucine (L) in the sequences may be substituted by Isoleucine (I).

**Figure 2.** Thin layer chromatogram of SPE**Figure 3.** Base peak ion chromatogram obtained by an MS^E data collection technique method (UPLC-qTOF/MSE) in positive mode. Of the SPE aqueous extract from seahorse.

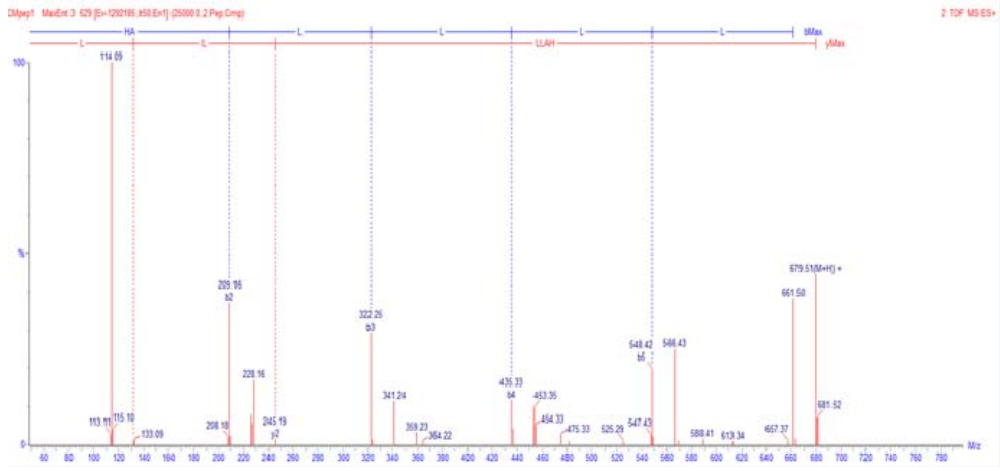
A) m/z 453 (peak 1)



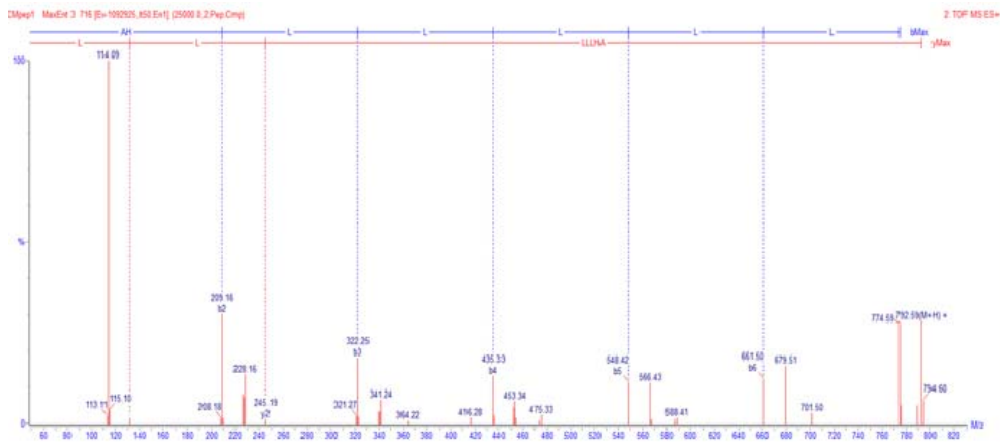
B) m/z 566 (peak 2)



C) m/z 679 (peak 3)



D) m/z 792 (peak 4)



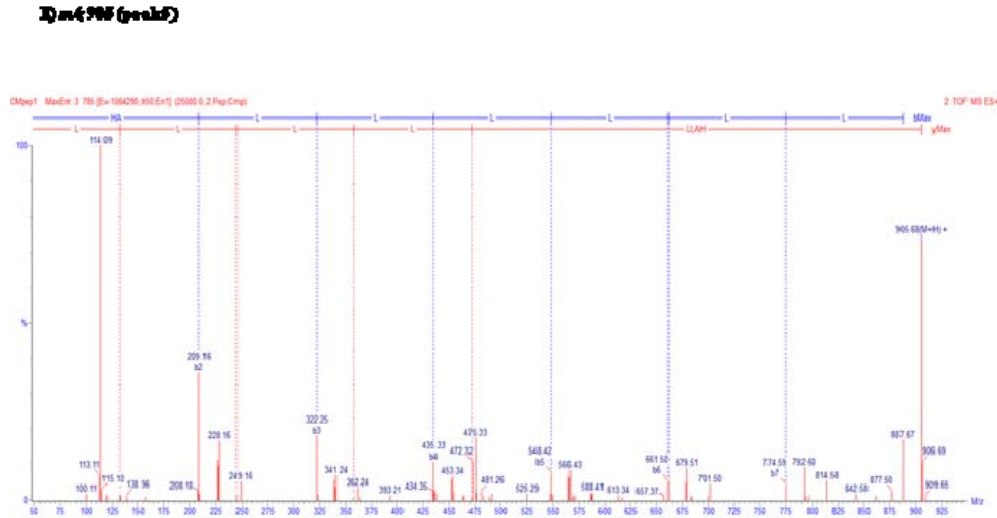


Figure 4. LC-MS^E results identifying the specific sequences for peptides. (A-E) MS^E fragment ions confirming the sequence of peptides. The peaks 1 m/z 453.34 [M+H]⁺, 2 m/z 566.43 [M+H]⁺, 3 m/z 679.51 [M+H]⁺, 4 m/z 792.59 [M+H]⁺ and 5 m/z 905.68 [M+H]⁺ corresponds to the sequences AHLL, AHLLL, AHLLLL, AHLLLLL and AHLLLLL, respectively. The amino acid Leucine (L) in the sequences may be substituted by Isoleucine (I).

Profile of the cytotoxicity of *H. reidi* hydroalcoholic extract on spleen cells cultures. Figure 5 shows that 2000, 1000 and 500 $\mu\text{g/mL}$ of *H. reidi* extract were toxic to mouse spleen cells in culture compared with the negative control (non-treated cells). However, no toxicity was detected using concentrations ranging from 250 to 7.8 $\mu\text{g/mL}$ compared with the negative control. Thus, we selected three concentrations of the *H. reidi* extract (125, 62 and 31 $\mu\text{g/mL}$) that do not have cytotoxic effect on spleen cells to test the inflammatory parameters in the present work.

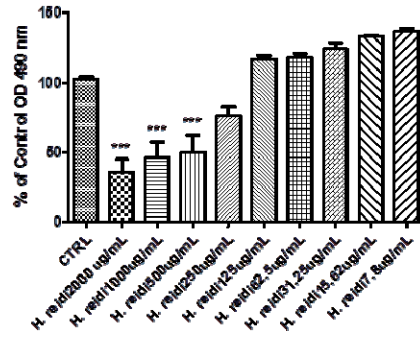


Figure 5: Cytotoxicity of *H. reidi* hydroalcoholic extract on mice spleen cells. The cells were cultivated with RPMI medium (CTRL) or RPMI medium containing concentrations of *H. reidi* extract from 2000 to 7.8 µg/mL and cultivated for 72 hours at 37°C in an atmosphere with 5% CO₂. *** p < 0.001 vs control; ANOVA-Tukey's test.

Effect of *H. reidi* hydroalcoholic extract on cytokine production. To determine the potential mechanisms associated with the effects of *H. reidi* on inflammatory processes, the levels of IL-4, IL-5, INF-γ and IL-10 were measured in the supernatants of cultured spleen cells. The results showed that PWM increased the levels of IL-4, IL-5, INF-γ and IL-10 compared with non-stimulated cultures (p < 0,001) (Fig. 6 A, B, C and D). Treatment with *H. reidi* extract decreased PWM-induced IL-4 production at all concentrations tested (125 and 62 µg/mL, p < 0.001 and 31 µg/mL, p < 0.1 (Fig. 6 A) as well as IL-5 production at 125 µg/mL (p < 0.001) and 62 µg/mL (p < 0.1) (Fig. 6 B). However, a significant decrease in the INF-γ levels (p < 0.001) was only observed at 125 µg/mL (Fig. 6 C). In addition, we observed a significant increase in IL-10 production at the highest concentration of the extract of *H. reidi* tested (125 µg/mL, p < 0.1) (Fig. 6 D).

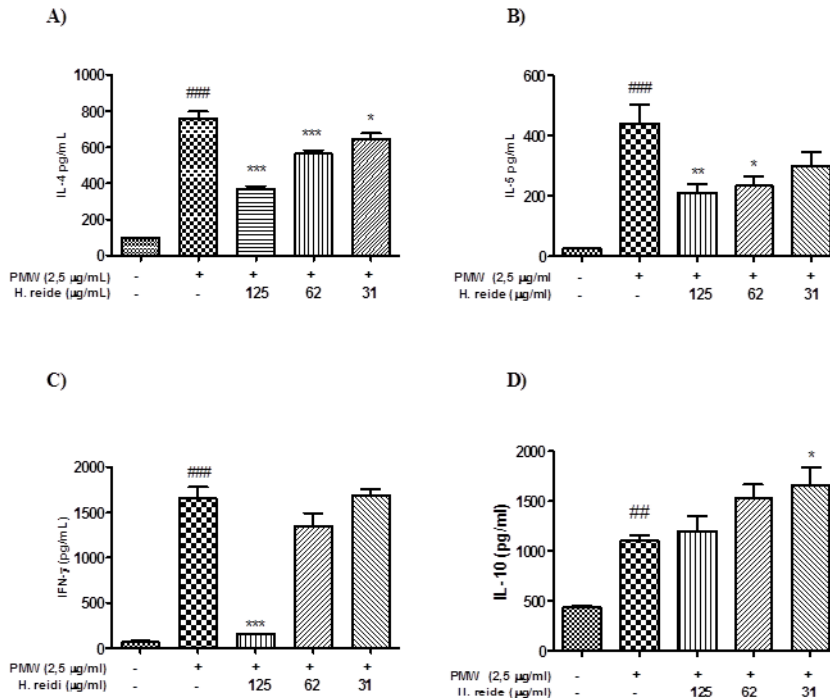


Figure 6. Effect *H. reidi* hydroalcoholic extract on IL-4, IL-5, INF- γ and IL-10 production in spleen cells stimulated with PWM. Spleen cells were incubated with 2,5 $\mu\text{g/mL}$ of PWM with or without *H. reidi* at concentrations of 125, 62 or 31 $\mu\text{g/mL}$. IL-4, IL-5, INF- γ and IL-10 quantification was conducted by sandwich ELISA. The columns represent the mean values of the results obtained from six animals, and the error bars represent the standard error of the means (### $p < 0.001$ vs control), ANOVA-Tukey's test.

***H. reidi* hydroalcoholic extract reduces NO production *in vitro*.** The NO production in peritoneal macrophages was also evaluated *in vitro*. Considerable levels of NO were detected in the supernatant of macrophages treated with 5 $\mu\text{g/mL}$ of LPS. Treatment with 125 and 62 $\mu\text{g/mL}$ of *H. reidi* extract $p < 0.001$ suppressed NO production in LPS-treated macrophages. The results are expressed as the means and standard deviation; ANOVA-Tukey's test (Fig. 7).

***In vivo* evaluation of anti-inflammatory activity.** Leukocyte (Fig. 8A) and neutrophil (Fig. 8B) counts in the peritoneal cavity significantly ($p < 0.001$) increased at 6 h after peritonitis induced by lipopolysaccharide (LPS) compared with corresponding counts in the I group. However, pretreatment with *H. reidi* extract (100 mg/Kg) reduced leukocyte migration compared with the (II) LPS group and produced a significant effect via a subcutaneous route (IV group). These effects were similar to those obtained after pre-treatment with dexamethasone (V group) at 0.3 mg/kg ($p < 0.001$). Differential cell counts showed a reduction of neutrophil migration via both routes tested compared with the I group, without difference with the dexamethasone-treated group ($P < 0.001$). The intraperitoneal injection of LPS (1 $\mu\text{g/mL}$) in mice caused a significant increase ($39.8 \pm 8.4 \times 10^5$ cells/mL; $p < 0.001$) in leukocyte migration to the peritoneal cavity compared to the I group (saline: $0.6 \pm 0.20 \times 10^5$ cells/mL; $p < 0.001$). Pre-treatment with *H. reidi* extract (100 mg/Kg) by subcutaneous route cause reduction in leukocyte migration compared with the II group ($0.82 \pm 0.13 \times 10^5$ cells/mL; $p < 0.001$) and by oral route ($12 \pm 3.3 \times 10^5$ cells/mL; $p < 0.001$) similar to those obtained after pre-treatment with dexamethasone at 0.3 mg/kg ($2.5 \pm 0.7 \times 10^5$ cells/mL; $p < 0.001$). Differential cell counts showed a reduction of neutrophil migration after pre-treatment with *H. reidi* extract at both routes compared with the control group (saline), without difference with the dexamethasone-treated group ($P < 0.001$).

Effect of *H. reidi* hydroalcoholic extract on IL-6 production in the peritoneal lavage. LPS administration produced a significant ($p < 0.001$) increase of the IL-6 levels in the peritoneal lavage compared with the control group (I). Pre-treatment

with *H. reidi* extract showed a significant reduction of the IL-6 concentration compared with the II group and without statistic difference with the dexamethasone-treated group (V) (fig. 9).

Effect of *H. reidi* hydroalcoholic extract on the stabilization of the erythrocyte membrane As shown in Figure 10, *H. reidi* extract at all concentrations (125, 62 and 31 $\mu\text{g/mL}$) protected the mice erythrocyte membrane against heat-induced lysis, as shown by the high percentage of haemolysis inhibition. The inhibition was statistically significant compared with the negative control ($p < 0.001$). Aspirin (AAS) was used as a standard drug in this assay, presenting a stabilizing action similar to the concentrations of *H. reidi*, compared with the negative control ($p < 0.001$).

***H. reidi* hydroalcoholic extract attenuates the NF- κ B expression in spleen cells cultures.** The nuclear factor NF- κ B pathway plays a seminal role in inflammation, leading to the expression of pro-inflammatory genes, including chemokines, cytokines, and adhesion molecules²⁰. In order to assess the potential mechanism of action of *H. reidi*, NF- κ B expression was assessed by RT-PCR. The results showed that PWM-stimulated splenocytes increased the expression of NF- κ B compared with control cultures $p < 0.001$ (Fig. 11). However, when the cells were stimulated with PWM and treated with *H. reidi* (125 $\mu\text{g/mL}$), the expression of NF- κ B was reduced ($p < 0.001$) compared with the positive control. The results were performed in triplicate. ANOVA-Tukey's test.

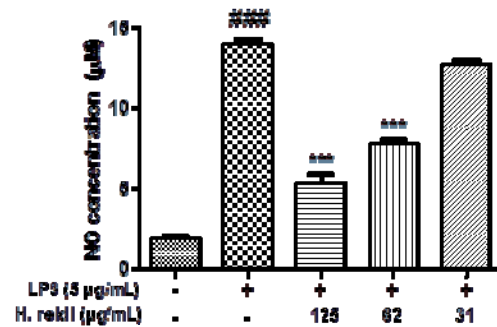


Figure 7. *H. reidi* hydroalcoholic extract decreases NO production in peritoneal macrophages. The following groups were presented: peritoneal macrophages not stimulated, stimulated with LPS or stimulated with LPS and treated with *H. reidi* (125, 62 and 31 $\mu\text{g/mL}$). (### $p < 0.001$ vs control group; *** $p < 0.001$ vs. LPS group), ANOVA-Tukey's test.

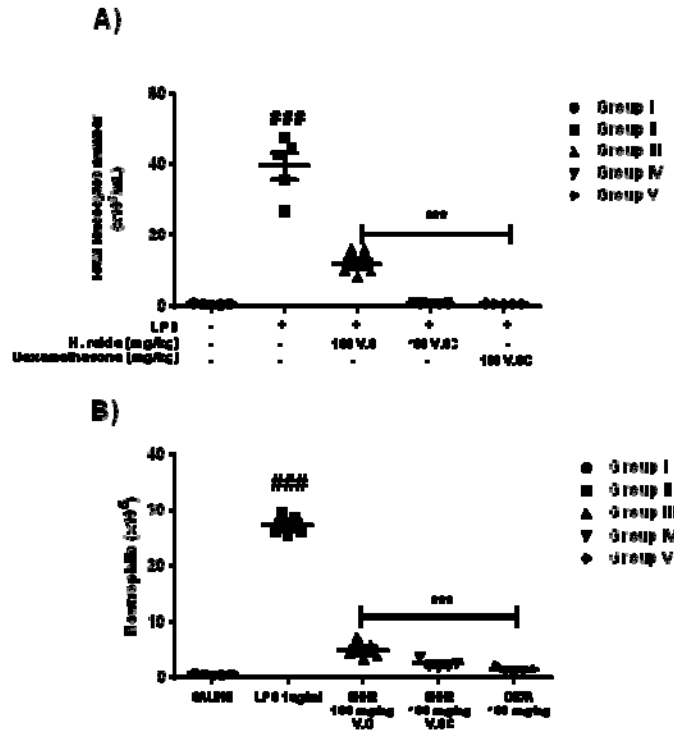


Figure 8. Effect of the oral administration of 0.9% sterile saline solution, *H. reidi* hydroalcoholic extract EHHR mg/kg, v.o (III group) or v.sc (IV group), and dexamethasone (DEXA 0.3 mg/kg - V group) on the total number of leukocytes present in the peritoneal lavage (A) and neutrophilia (B) of male mice with LPS-induced peritonitis (1 µg/kg LPS/0.2 mL/cavity – II group). Each point represents a mean of five animals, and the error bars represent the standard deviation from the means. ### p < 0.001 vs control (I group) and *H. reidi* tested concentrations were compared with the (II group) ***p < 0.001. ANOVA-Tukey's test.

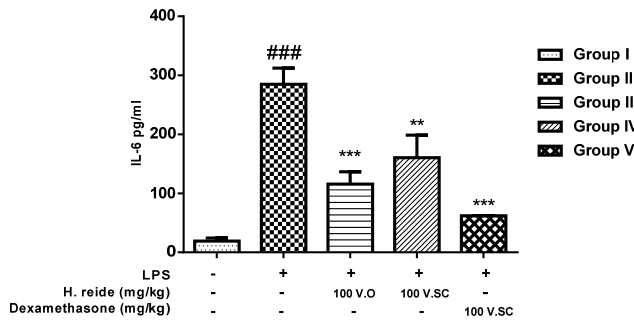


Figure 9. Effect of the oral administration of 0.9% sterile saline solution, *H. reidi* hydroalcoholic extract EHHR mg/kg, v.o (III group) or v.sc (IV group), and dexamethasone DEXA 0.3 mg/kg (V group) on the concentration of IL-6 in the peritoneal lavage of male mice with LPS-induced peritonitis (1 µg/kg LPS/0.2 mL/cavity – II group). The columns represent the mean values of the results obtained from five animals, and the error bars represent the standard deviation from the means. ### p < 0.001 vs control (I group). ANOVA-Tukey's test.

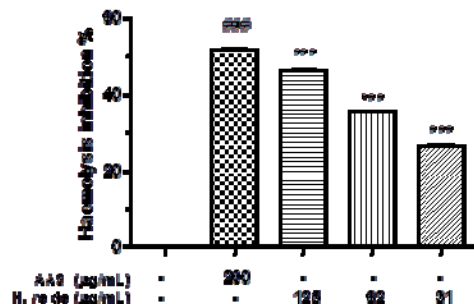


Figure 10: Effect of *H. reidi* hydroalcoholic extract on stabilization of erythrocyte membrane. Aspirin, as a standard drug, was compared with the negative control group ### p < 0.001, and *H. reidi* tested concentrations were compared with the negative control group *** p < 0.001. The results are expressed as the mean and standard deviation, ANOVA-Tukey's test.

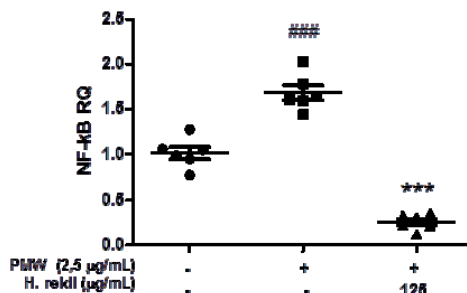


Figure 11. Effect of *H. reidi* hydroalcoholic extract on NF-κB expression in spleen cells cultures. The cells were exposed or not to PWM (2.5 µg/mL) and treated or not with *H. reidi* extract (125 µg/mL) for 72 hours at 37°C and 5% CO₂. The results were expressed as the mean and standard deviation, ANOVA-Tukey's test.

DISCUSSION

Natural products have historically been used to treat inflammatory conditions such as asthma, arthritis and gastritis²¹. In the present study, we examined the *in vitro* and *in vivo* anti-inflammatory potential of the marine species *Hippocampus reidi* (*H. reidi*). *H. reidi* has commonly been used in folk medicine for the treatment of inflammatory disorders, including gastritis, asthma, arthritis and kidney disorders²². Studies on the extracts of another species of *Hippocampus* (*Hippocampus kuda*) have demonstrated antioxidant and antiasthmatic activities^{8, 23}. Two independent ethnobotanical surveys, describing plant and animal species in folk medicine to treat asthma and other inflammatory conditions²⁴, identified *Hippocampus reidi* as the animal species used to treat inflammatory diseases, particularly asthma. Currently, however, no scientific study has confirmed this potential.

In recent years, there has been much interest in identifying a powerful, nontoxic, and natural anti-inflammatory therapeutic agent from natural resources to reduce inflammatory responses. Marine resources are vast and varied and have unique bioactivities because of the diverse environment. In the present study, we successfully showed that a *H. reidi* hydroalcoholic extract suppresses the inflammatory response both *in vitro* and *in vivo*. In this study, we isolated an active mixture of five peptides from seahorse and the amino acid sequence was tentatively identified using high-resolution mass values. Further analysis will be conducted to certify which amino acid is present in the sequence, leucine and isoleucine, since such amino acids have the same molecular weight. To the best of our knowledge, this is the first report of peptides in *H. reidi* extracts. The presence of such molecules in *Hippocampus* extracts is consistent with previous studies that have evaluated the anti-inflammatory potential of peptides from *Hippocampus*^{7, 23}.

The treatment with *Hippocampus reidi* hydroalcoholic extract shows various biological properties, decreasing some *in vitro* inflammatory parameters, such as IL-4, IL-5 and INF- γ production, indicating the attenuation of both Th1 and Th2 inflammation as well as the up-regulation of the immunoregulatory cytokine, IL-10. We also observed the reduction of nitric oxide production as well as the stabilization of the erythrocyte membrane, in addition to the decreased expression of the transcription factor, NF-kB. The production of IL-5 and IL-4 from PWM-stimulated splenocytes was remarkably inhibited by the addition of *H. reidi*. Th2 cytokines, including IL-4 and IL-5, play an important role in the pathogenesis of allergic inflammation²⁵. IL-4 promotes the proliferation and differentiation of Th2-type T cells and the production of IgE antibodies. IL-5 is associated with the maturation, activation and recruitment of eosinophils²⁶. The down-regulation of Th2 cytokine constitutes an important strategy for treating allergic inflammatory diseases, and in this way, additional *in vivo* studies are needed to address this idea. IFN- γ has been recognized as a signature proinflammatory cytokine that plays a central role in autoimmune disease and inflammation. In this context, IFN- γ upregulates a variety of pro-inflammatory parameters such as interleukin IL-12, macrophage activation and consequently, nitric oxide production²⁷. The highest concentration of *H. reidi* extract (125 $\mu\text{g/mL}$) significantly decreased the INF- γ levels in cultured spleen cells. In addition to IL-4, IL-5 and INF- γ , IL-10 has been up-regulated by *H. reidi* extract. IL-10, an anti-inflammatory cytokine, is described to modulate both Th1 and Th2 immune responses²⁸. These results suggest that IL-10 may play a role in decreasing IL-4, IL-5 and INF- γ induced by *H. reidi* extract treatment *in vitro*. This immunomodulatory property of *H. reidi* extract may be due to the decreased expression of NF-kB as described in the present study. Previous studies have also shown that extracts from another species of *Hippocampus* (*Hippocampus kuda*) reduce the production of inflammatory cytokines by inhibiting the activation of NF-kB and the transcription of inflammatory proteins^{7,8}. Furthermore, the *H. reidi* extract reduced the NO production by peritoneal macrophages stimulated for LPS. This result is consistent with those of previous studies attributing the anti-inflammatory properties of natural products to the reduction of NO production by iNOS, which has been associated with NF-kB inhibition.²⁹ *H. reidi* extract also demonstrated anti-inflammatory effects *in vivo*, reducing the total number of leukocytes and neutrophils in the peritoneal lavage after LPS-induced peritonitis. During LPS exposure, different pathways are activated and induce the production of proinflammatory cytokines, including IL-6³⁰. These proinflammatory agents play a crucial role in the elicitation of inflammatory reactions and have been associated with the pathogenesis of various autoimmune and chronic inflammatory diseases³¹. Similar to dexamethasone, *H. reidi* extract also inhibited the IL-6 production in the LPS-induced peritonitis.

The lysosomal enzymes released during inflammation produce several consequences for the organism³. In this study, we used haemolysis assays to assess the membrane stabilization properties to *H. reidi* extract. According to Mounnissamy and collaborators³², the capacity of non-steroidal drugs inhibiting lysosomal enzymes is due to the stabilization of the membrane and subsequent inhibition of the release of inflammatory mediators. These results suggested that *H. reidi* hydroalcoholic extract was effective in inhibiting heat-induced haemolysis at different concentrations, comparable to the standard drug Aspirin. These data may explain, at least in part, the observed anti-inflammatory activity of *H. reidi*.

In conclusion, this study is the first to demonstrate the anti-inflammatory activity of *Hippocampus reidi* hydroalcoholic extract *in vitro* and *in vivo*. Further studies must be conducted to investigate this effect *in vivo* and identify the molecules responsible for this property.

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