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Antihelmintic effects of nutmeg (*Myristica fragans*) on *Anisakis simplex* L3 larvae obtained from *Micromesistius potassou*

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ABSTRACT

Anisakis simplex is a foodborne pathogen that can produce human infections and allergic reactions due to the high consumption of raw fish. The seeds of *Myristica fragans* (Myristicaceae), popularly known as nutmeg, are worldwide used as a culinary spice due to its flavour and properties in food preservation. A nutmeg extract was prepared, analyzed, screened for cytotoxicity and tested against *Anisakis simplex* L3 larvae. In order to detect the biologically active constituents of the extract, myristicin was tested on the larvae. An acetylcholinesterase inhibition bioassay was also carried out to investigate the antihelmintic mechanism of action. Our results demonstrate that nutmeg exerts antihelmintic effects on *Anisakis simplex*, being myristicin one of the active compounds. The extract induced a high rate of dead anisakis at concentrations between 0.5 and 0.7 mg/ml without being considered cytotoxic; however, an inhibition of acetylcholinesterase was discarded as the molecular mechanism involved in the activity.

1. Introduction

Bacteria, fungi and parasites are leading causes of deterioration of food quality and human risks (Magnusson et al., 2012). Due to the increase and the worldwide acceptance of "sushi" and other traditional raw foods, the human parasitic infection of the gastrointestinal tract caused by *Anisakis simplex*, known as anisakiasis, has become a big issue in the areas of food safety and public health (Madrid et al., 2012). *Anisakis simplex* is a parasitic nematode that hosts marine mammals like dolphins and whales but also small fishes. Humans usually eat L3 larvae hidden in raw and undercook fish and cephalopod (Chai et al., 2005; Hochberg and Hamer, 2010). Although anisakiasis is sometimes misdiagnosed with other gastrointestinal diseases this parasite causes a high number of allergic reactions with urticaria and anaphylaxis as clinical symptoms (Audicana and Kennedy, 2008). The best protection against anisakiasis may be educating people about the risks of eating raw fish and recommending cooking at temperatures over 60 °C. In the European Union, the legislation establishes that certain fishery products must be frozen at a temperature of not more than –20 °C in all parts of the product for not less than 24 hours in order to avoid parasites (EU, 2004). However, many other countries lack of regulation on food hygiene.

In the attempt to reduce the use of chemicals due to the concern about human health and environmental toxicology, new and safer food control approaches such as the use of natural products are nowadays being developed (Abushelaibi et al., 2012; Chen et al., 2012; Davidson et al., 2013; Juneja et al., 2012; Negi, 2012).

Myristica frangans (Myristicaceae) is a tree native to Indonesia, being nowadays cultivated in other Asian countries and in the Caribbean, whose seeds are popularly known as nutmeg and worldwide used as a spice in different types of cuisine (Vangils and Cox, 1994). Although the main value of nutmeg may be as an ingredient in foods due to its flavour and preservative properties, this species is used in traditional medicine for the treatment of intestinal diseases like colitis in some Asian countries (Jazayeri et al., 2014; Kim et al., 2013; Touwaide and Appetiti, 2013).

There are several papers reporting the chemical constituents and the biological effects of nutmeg. Nutmeg contains fatty acids, lignans, terpenes and phenylpropane derivatives such as myristicin, elemicin and safrol (Choo et al., 1999; Kapoor et al., 2013; Moreira Valente et al., 2011; Piaru et al., 2012; Simpson and Jackson, 2002).

Common activities attributed to nutmeg extracts are antioxidant, antimicrobial, cytotoxic and anti-inflammatory effects (Jin et al., 2012; Olajide et al., 1999; Phuong Thien et al., 2014; Piaru et al., 2012; Shafiei et al., 2012; Sulaiman and Ooi, 2012). Antiparasitic properties have also been reported (Pillai et al., 2012) and the effects of nutmeg essential oil in *Anisakis* larvae have been studied in an early work (Oishi et al., 1974).

In this study, we have prepared, analyzed, screened for cytotoxicity and tested against *Anisakis* L3 larvae a nutmeg ethanolic extract. Authors have also investigated a possible mechanism of

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action in terms of acetylcholinesterase inhibition because this enzyme plays a key role in neuromuscular contraction of nematodes.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and myristicin were supplied by Sigma-Aldrich. Solvents were acquired through a common supplier.

2.2. Plant material and extract preparation

Ground nutmeg was acquired in a local supermarket in the form of a manufactured product. Approximately, 20 g of nutmeg was extracted with 250 ml of ethanol at 60 °C for 40 min. The extract was filtered and the solvent was removed to dryness in a rotary evaporator (Büchi R-114, Switzerland). The dry extract was kept at –20 °C until use.

2.3. Phytochemical analyses by chromatographic techniques

2.3.1. Gas chromatography–mass spectrometry (GC–MS) analysis of nutmeg extract

The extract was analyzed by GC–MS on an Agilent 6890N Network GC system coupled to a 5973 Network Mass Selective Detector, accelerating voltage –69.9 eV, recoding masses of 35.00–400.00. GC conditions: injector temperature: 150 °C; temperature programme: start 50 °C, 20 °C/min to 300 °C; column: HP5MS (5% phenylmethylsiloxane) capillary, 30.0 m × 250 µl × 0.25 µm nominal. Carrier gas: helium at 1.0 ml/min. A NIST library was used for comparison of MS data.

2.3.2. Detection of myristicin by thin layer chromatography (TLC)

One hundred micrograms of our extract and commercial myristicin (as reference) was applied to Merck silica-gel 60_{F254} plates and eluted in toluene:ethyl acetate (93:7). The plate was observed at 366 nm. The same plate was then spayed with vanillin–sulphuric acid, heated at 100 °C and observed at visible.

2.4. Cytotoxicity of the extract in adherent epithelial cells

The cytotoxic effect of the extract was screened through the MTT assay (Mossman, 1983) using HeLa cells in order to determine the doses that should be assayed against *Anisakis*. Cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin–glutamine. Cultures were incubated in the presence of 5% CO_2 at 37 °C and 100% relative humidified atmosphere. Cells were seeded in 96-well microplates at a density of 7×10^3/well and grown for 48 h at 37 °C. Cells were then treated with various concentrations of nutmeg extract (0.01–0.7 mg/ml) for 24 h and a MTT solution was added and incubated for 4 h at 37 °C. Cell survival was measured as reduction of MTT into formazan at 550 nm in a microplate reader. Three different plates were done to screen the cytotoxicity of the extract.

2.5. In-vitro larvicidal activity

Anisakis simplex L3 larvae were isolated from the intermediary host *Micromesistius potassou* (blue whiting) purchased from several fish markets in Zaragoza. Worms were washed several times on sterile solution of 0.9% NaCl and identified under light microscope according to morphological features. Only larvae with length >2.0 cm were used. Ten larvae were introduced in each well of polystyrene plates with 2 ml of sterile saline solution containing different concentrations of the test solutions (Gómez-Rincón et al., 2014). The nutmeg extract was tested against anisakis in the range of 0.1–0.7 mg/ml. Myristicin was tested at three different concentrations (0.1, 1, 2 mg/ml). The parasites were incubated at 37 °C in 5% CO_2. Levamisole was used as the reference antiparasitic drug. Larvae were examined at 24 h and 48 h under microscope and immobile L3 were considered dead. LD_{50} for nutmeg extract was calculated using non-linear regression (GraphPad Prism 5).

2.6. Inhibition of acetylcholinesterase

The inhibition of acetylcholinesterase (AChE) was determined in 96-microplates by the Ellman method with some modifications. Each well contained 25 µl of 15 mM ATCI in Millipore water, 125 µl of 3 mM DTNB in buffer C (50 mM Tris–HCl, pH 8, 0.1 M NaCl, 0.02 M $MgCl_2$ 6 H_2O), 50 µl buffer B (50 mM Tris–HCl, pH 8, 0.1% bovine serum), 25 µl of test compound. Every concentration tested of nutmeg, myristicin and levamisole (used as reference) was diluted in DMSO and tested in triplicates. Then, 25 µl 0.22 U/ml AChE was added and the absorbance was measured eight times every 13 s at 405 nm.

2.7. Statistical analyses

All experiments with *Anisakis simplex* larvae were performed in triplicates in at least three different weeks. Data are presented as means ± standard error and results were statistically analyzed using GraphPad Prism version 5. One way ANOVA followed by Dunnett's test was used to detect significant differences.

3. Results

3.1. Phytochemical analyses by chromatographic techniques

The GC–MS analysis of nutmeg extract showed the presence of four main compounds: myristicin (64.5%), myristic acid (18.7%), terpinen-4-ol (8.8%) and methoxyeugenol (8.1%) (Fig. 1).

The presence of myristicin was also confirmed by TLC, being detected at 254 nm as well as at visible light after spraying the plate with the reagent vanillin–sulphuric acid and heated at 100 °C.

3.2. Cytotoxicity of the extract in adherent epithelial cells

The cytotoxicity of the extract is shown in Fig. 2. We observed that the higher concentration of the extract reduced cell survival (63% cell viability); therefore, we considered 0.7 mg/ml the maximum concentration for the antihelmintic assay against *Anisakis*. Lower doses were considered as non-cytotoxic.

3.3. In-vitro larvicidal activity

As part of our ongoing studies with *Anisakis*, we detected a prevalence of 100% (n = 100) on the species *Micromesistius potassou* (blue whiting) with a high variability of larvae per fish.

Fig. 3 shows the effect of nutmeg extract against living *Anisakis simplex* L3 larvae obtained from raw fish in the range of 0.1–0.7 mg/ml after 24 and 48 hours of exposition. The highest tested concentrations produced a significant mortality rate of 92%. LD_{50} values for nutmeg were calculated by non-linear regression (0.5 and 0.47 mg/ml at 24 and 48 hours respectively).

Due to the fact that myristicin was detected as one of the main constituents in the extract, its effect was studied on the anisakis larvae at three different concentrations (Fig. 4). All tested doses of myristicin induced death to larvae compared to control wells (non-treated larvae) which means that this compound is one of the active ingredients in relation with the nematicidal activity of nutmeg. Levamisole, tested at 0.1 mg/ml, induced 100% of larvae death after 24 h.