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Protective effect of *Heliotropium foertherianum* (Boraginaceae) folk remedy and its active compound, rosmarinic acid, against a Pacific ciguatoxin

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ABSTRACT

Ethnopharmacological relevance: Senescent leaves of *Heliotropium foertherianum* Diane & Hilger (Boraginaceae) are traditionally used in the Pacific region to treat Ciguatera Fish Poisoning. This plant contains rosmarinic acid that is known for its multiple biological activities. In the present study, *H. foertherianum* aqueous extract, rosmarinic acid and its derivatives were evaluated for their capacity to reduce the effect of ciguatoxins.

Materials and methods: Aqueous extract of *H. foertherianum* leaves was prepared and studied for its effects against a Pacific ciguatoxin (P-CTX-1B) in the neuroblastoma cell assay and the receptor binding assay. Rosmarinic acid and six derivatives were also evaluated by means of these bioassays. For this purpose, we have developed an improved synthetic route for caffeic acid 3,4-dihydroxy-phenethyl ester (CADPE).

Results: Both the aqueous extract of *H. foertherianum* leaves and rosmarinic acid showed inhibitory activities against a Pacific ciguatoxin in the above bioassays. Among all the molecules that were evaluated, rosmarinic acid was the most active compound.

Conclusion: These results confirm further the potential of *H. foertherianum* in the treatment of Ciguatera Fish Poisoning.

1. Introduction

Ciguatera Fish Poisoning (CFP) is a seafood intoxication caused in humans by the consumption of tropical coral reef fishes that have accumulated ciguatoxins (CTXs) in their tissues (Lewis, 2001). Produced by *Gambierdiscus* dinoflagellates, CTXs are potent polyether neurotoxins that bioaccumulate and biotransform in fish tissues up the trophic chain (Yasumoto, 2005). CTXs have high affinity to site 5 of the voltage-gated sodium channel (VGSC). Using the receptor binding assay (RBA) and neuroblastoma cytotoxicity assay (NCA), their mode of action is applied to detect and quantify them (Caillaud et al., 2010), as well as to carry out pharmacological studies (Boydron-Le-Garrec et al., 2005).

Though mortality due to CFP is relatively low, the morbidity is significant with an important socioeconomic impact on island populations (Davis Lewis, 1983) with 50,000–100,000 cases listed annually. The symptoms of CFP include a combination of gastrointestinal and neurological symptoms and, in severe cases, cardiovascular problems (Bagnis et al., 1979).

At present, the intravenous injection of hypertonic mannitol is the most widely studied treatment for CFP (Friedman et al., 2008). However mannitol needs to be given within 72 h of the first signs of intoxication in order to observe a significant improvement in the patient's state (Palafox et al., 1988). Furthermore, this treatment requires caution as it causes a further loss of fluids in CFP patients (Nicholson and Lewis, 2006). A recent study, in the prospect of a treatment of CFP, is interested in brevenal, a polyether produced by dinoflagellate *Karenia brevis*. This molecule appears to be an antagonist to brevetoxin, a marine toxin possessing structural and pharmacological similarities to CTXs, in RBA (Bourdelais et al, 2004).

In the Pacific, numerous traditional herbal remedies are preferentially used to treat CFP (Bourdy et al., 1992; Laurent et al.,

Abbreviations: CADPE, caffeic acid 3,4-dihydroxy-phenethyl ester; CAPE, caffeic acid phenethyl ester; CFP, Ciguatera Fish Poisoning; CTXs, ciguatoxins; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; NCA, neuroblastoma cytotoxicity assay; O, ouabain; PbTx, brevetoxin; RBA, receptor binding assay; RPMI, Roswell Park Memorial Institute medium; V, veratridine; VGSC, voltage-gated sodium channel

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1993; Kumar-Roiné et al., 2011). Several plant species have shown their efficacy in various bioassays and *Vitex trifolia* L., *Euphorbia hirta* L. and *Heliotropium foertherianum* Diane & Hilger have attracted attention because of their activities (Benoit et al., 2000; Boydron-Le-Garrec et al., 2005; Kumar-Roiné et al., 2009; Matsui et al., 2009). Among these plants, *H. foertherianum*, a common coastal plant of the Boraginaceae family previously named *Argusia argentea* (L.f.) Heine, is the most widely used in Pacific islands to treat CFP. Its use has been reported in New Caledonia, Vanuatu, French Polynesia, Tonga, Micronesia, and in the Ryukyu Islands in Japan (Kumar-Roiné et al., 2011). *H. foertherianum* is also known as an antidote against jellyfish in Japan. Recent studies have shown that *H. foertherianum* inhibits snake venom-induced hemorrhage, and that this property is due to the presence of rosmarinic acid, isolated from the plant (Aung et al., 2010). In order to understand the activity of *H. foertherianum* against CFP, its aqueous leaf extract was evaluated by RBA with bioguided fractionation, which led to the isolation of rosmarinic acid (Kumar-Roiné, personal communication).

Rosmarinic acid (Fig. 1), an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid derived from hydroxycinnamic acid, is a polyphenolic compound. This molecule is well-known with a multitude of biological activities such as antiviral, antibacterial, anti-inflammatory (Osakabe et al., 2004b; Swarup et al., 2007; Gamaro et al., 2011), antioxidant and anti-mutagen (Petersen and Simmond, 2003). Known to reduce hypotension (Bult et al., 1985), rosmarinic acid also possesses hepatoprotective (Renzulli et al., 2004), cytoprotective (Lee et al., 2008), neuroprotective (Fallarini et al., 2009) and anti-acetyl cholinesterase properties (Falé et al., 2009). Exhibiting very low toxicity with a LD_{50} in mice of 561 mg/kg after intravenous application (Parnham and Kesselring, 1985), rosmarinic acid is rapidly eliminated from human and rat blood circulation after oral administration and is metabolized predominantly to caffeic acid, coumaric acid and ferulic acid (Fig. 2; Baba et al., 2005). Rosmarinic acid is already commercialized via cosmetic or food complement preparations with daily doses and day or week-long intake. This substance is such of interest that biotechnological production by plant *in vitro* cultures has been proposed (Petersen and Simmond, 2003).

In our continuing efforts to assess the efficacy of *H. foertherianum* in the treatment of CFP and the role that rosmarinic acid could play in this remedy, both *H. foertherianum* aqueous leaf extract and rosmarinic acid have been investigated in NCA with a Pacific ciguatoxin (P-CTX-1B), the most potent CTXs analog, and in RBA with brevetoxin-3 (PbTx-3). In order to show rosmarinic acid's specificity of action, we also report herein the activity in these bioassays of six compounds that are close to rosmarinic acid in terms of their chemical structure.

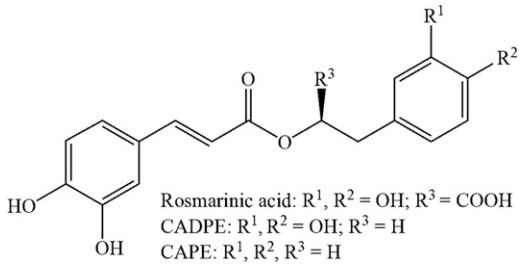

Fig. 1. Chemical structures of rosmarinic acid, caffeic acid 3,4-dihydroxyphenethyl ester (CADPE) and caffeic acid phenethyl ester (CAPE).

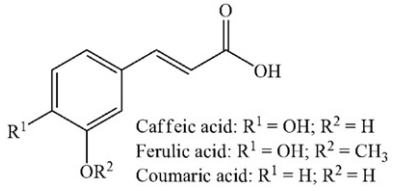

Fig. 2. Chemical structures of caffeic acid, ferulic acid and coumaric acid.

2. Materials and methods

2.1. Chemicals and reagents

P-CTX-1B and PbTx-3 was provided by the Institut Louis Malardé (ILM, French Polynesia). Rosmarinic acid, caffeic acid, coumaric acid, ferulic acid, caffeic acid 3,4-dihydroxy-phenethyl ester (CAPE), curcumin, ouabain (O), veratridine (V), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), dimethyl sulfoxide, kits tox-4 and tox-7 were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). All the cell culture reagents were purchased from Invitrogen. HPLC grade solvents were from Merck. Synthesis grade solvents were from Bioblock-Fisher scientific (Illkirch, France). Anhydrous methanol and tetrahydrofuran were obtained after distillation over calcium hydride (Sigma-Aldrich, Saint Quentin Fallavier, France).

2.2. Plant extract preparation

Senescent leaves of *H. foertherianum* were collected in Tubuai (Australes Archipelago, French Polynesia) in January 2011. The sample was identified by comparison to fully registered specimen (identification number: Florence J. 6049) kept at the herbarium of French Polynesia. Following the traditional recipe (Laurent et al., 1993), the leaves (50 g) were boiled for 30 min in distilled water (1 liter) and the filtrate was freeze-dried and stored at 4 °C until further use.

2.3. Rosmarinic acid HPLC–UV dosage in the plant extract

Analyses were performed using a HPLC Kontron equipped with an UV detector and a Shodex Asahipak ODP-50 4D C18 (150 mm × 4.6 mm, 5 μm) column. Rosmarinic acid dosage was achieved with 0.01% trifluoroacetic acid in water (A) and acetonitrile (B) isocratic elution with 35% B for 10 min followed by a gradient elution from 35% to 45% B for 10 min. The flow rate was 1 ml/min, aliquots of 10 μl were injected and the absorbance was recorded at 320 nm. The dosage of rosmarinic acid in the plant extract was achieved with an external calibration method. Briefly, a standard solution was prepared with purchased rosmarinic acid and diluted into five concentrations within the range 20 μg/ml–100 μg/ml in order to construct a calibration curve with peak areas against concentrations. The *H. foertherianum* aqueous extract was prepared at 1 mg/ml. The products were dissolved in acetonitrile and 0.01% trifluoroacetic acid (50:50), ultrasonicated during 5 min, and filtered at 0.22 μm before being injected in the HPLC system. The rosmarinic acid peak on the *H. foertherianum* aqueous extract chromatogram was identified by its retention time and its UV spectrum. Finally, the area value of the rosmarinic acid peak on the *H. foertherianum* aqueous extract chromatogram was extrapolated on the calibration curve in order to estimate the content of rosmarinic acid in *H. foertherianum* aqueous extract.

2.4. Neuroblastoma cytotoxicity assay

2.4.1. Cell culture maintenance and seeding of neuroblastoma cells

Mouse neuroblastoma cells (Neuro-2A, CCL-131 line obtained from ATCC) were cultured in RPMI 1640 medium, supplemented