Biochemical and pharmacological characterization of toxins obtained from the fire coral *Millepora complanata*

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Abstract

*Millepora complanata* is a normal resident of coral reefs in the Mexican Caribbean. In this study, we describe for the first time the vasoconstrictor, phospholipase A2 (PLA2), and hemolytic activities elicited by a crude extract obtained from *M. complanata*. This extract caused a concentration-dependent contraction of isolated rat aortic rings (EC₅₀ = 22.4 ± 1.1 μg protein/mL). This effect was endothelium independent and significantly reduced in the absence of extracellular Ca²⁺ and when the intracellular Ca²⁺ stores were depleted. In addition, the crude extract obtained from *M. complanata* showed PLA2 activity (7.231 ± 0.092 mmol min⁻¹ mg⁻¹) and hemolysis of rat erythrocytes (HU₅₀ = 1.64 ± 1.04 μg protein/mL). The hemolysis increased in the presence of Ca²⁺ and decreased in the presence of cholesterol. Furthermore, this hemolysis was significantly reduced after incubation with an inhibitor of PLA2 enzymes. The hemolytic and vasoconstrictor effects were abolished after incubating the extract under denaturing conditions. Reverse phase chromatography of the *M. complanata* extract afforded 19 fractions (F1 to F19). F4 induced hemolysis and contained mainly a protein of 30 kDa, probably a PLA2 enzyme, while F8 and F11, containing mainly proteins of 15 and 20 kDa respectively, produced vasoconstrictor effects mediated by different mechanisms of action.

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1. Introduction

The genus *Millepora* comprises calcareous hydroids that are abundant in most coral reefs around the world (Lewis, 1989). These organisms belong to the phylum Cnidaria (class Hydrozoa) and, like other members of this phylum, possess stinging cells named cnidocytes, which contain specialized organelles known as nematocysts. These organelles are filled with toxins and discharge their content upon an appropriate stimulation (Tardent, 1995). Nematocysts of *Millepora* species can puncture the human skin and cause painful stings (Sagi et al., 1987; Bianchini et al., 1988). For this reason, hydrozoan corals are also commonly known as “fire corals”.

Biochemical and toxicological investigations of cnidarians of the genus *Millepora* have shown that their toxins are proteins. Early studies of *M. dichotoma* and *M. alcicornis* demonstrated that the crude extracts of these fire corals, obtained by soaking hydrocoral fragments in Sörensen buffer (pH 7, 4 °C), are highly toxic and display hemolytic activity in mice (Middlebrook et al., 1971; Wittle et al., 1971). Subsequently, a partially purified protein responsible for the lethal effects in mice was obtained from the nematocysts of *M. tenera* (Wittle et al., 1971). In another study, it was found that *M. platyphylla* and *M. dichotoma* nematocyst venoms displayed hemolytic, vasopermeable, and demonecrotic properties (Radwan, 2002). Recently, a hemolytic protein (32.5 kDa) with phospholipase A₂ (PLA₂) activity has been detected in *M. platyphylla* venom...
by ion exchange chromatography. This protein was named Milleporin-1 (Radwan and Aboul-Dahab, 2004).

In a previous study of Millepora complanata, a regular resident of coral reefs in the Mexican Caribbean (Lewis, 1989), it was found that the crude extract of this hydrocoral exhibited a concentration-dependent stimulation of spontaneous contractions of the guinea pig ileum (Rojas et al., 2002). In addition, chromatographic fractionation of the M. complanata extract, using FPLC size-exclusion chromatography, afforded 2 bioactive fractions, one of which contained proteins with molecular weights ranging from 17 to 44 kDa, and the other contained peptides with molecular weights less than 1.8 kDa. The contractions induced by the bioactive fractions were mainly caused by a direct action of the protein toxins on smooth muscle cells (Rojas et al., 2002).

The results of these previous studies indicated that species of the genus Millepora produce hemolytic proteins with properties that include a PLA2 activity, as well as other proteins that induce contractile effects on intestinal smooth muscle cells. At present it is not known whether the hemolytic toxins contained in Millepora venoms have other mechanisms of action in addition to those attributed to PLA2 activity. It is also unknown if these hemolysins are responsible for the smooth muscle excitatory effect.

In the present paper, we report for the first time the vasoconstrictor, PLA2, and hemolytic activities elicited by the crude extract obtained from M. complanata. The proteins responsible for these effects are found in different chromatographic fractions, suggesting that the venom of this hydrocoral contains hemolysins with PLA2 activity, as well as other proteins which modify vascular smooth muscle tone. The results derived from this study offer additional information about the toxins that M. complanata synthesizes in order to obtain food and to defend itself against predators.

2. Materials and methods

2.1. Specimen collection and crude extract preparation

M. complanata fragments were collected from coral reefs along the coasts of Puerto Morelos, Quintana Roo, México, at depths of 1–10 m. The fragments were immediately frozen in dry ice and transported to the laboratory where extraction was carried out.

Nematocyst discharge was accomplished by stirring the hydrocoral fragments in deionized water (pH 7) at 4 °C for 18 h. The extract obtained was centrifuged at 1000 × g for 15 min at 4 °C. This procedure was repeated twice, and the supernatant was lyophilized and stored at −20 °C. The lyophilized supernatant was used to determine biological effects. Protein content was measured using the Bradford method (Bradford, 1976).

2.2. Bioassays

2.2.1. Isolated rat aortic ring assay

Male Wistar rats (275–325 g) were anesthetized with chloroform and sacrificed by decapitation. The descending thoracic aorta was removed and placed in ice-cold, oxygenated Krebs–Henseleit solution (126.8 mM NaCl, 5.9 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 30 mM NaHCO3, and 5 mM D-glucose, pH 7.4) and immediately flushed with Krebs–Henseleit solution to prevent intravascular clot formation. The aorta was dissected free of adipose and connective tissue and cut into rings at 4- to 5-mm intervals. The aortic rings were mounted between stainless steel hooks and suspended in water-jacked, 7-mL organ baths containing oxygenated (95% O2 and 5% CO2) Krebs–Henseleit solution at 37 °C. The tissues were allowed to equilibrate for 60 min under a resting tension of 1.5 g. During this period, the bathing medium was exchanged every 15 min. After final adjustment of the passive resting tension to 1.5 g, aortic segments were contracted with 100 mM KCl. Once a stable contractile tone was reached, the bathing medium was exchanged to restore a resting tension of 1.5 g. After that, the tissues were contracted with 1 µM L-phenylephrine, the force of contraction was recorded, and this contraction was set as 100%. The bathing medium was exchanged again to restore a resting tension, and then the extract or the fractions were added to the organ bath. The isometric tension was measured by a Grass FT03 force-displacement transducer attached to a Grass 7D polygraph. The responses were expressed as a percentage of the initial contraction achieved with phenylephrine.

Preliminary pharmacological characterization was carried out to determine the mechanism of action involved in the vasoconstrictor effect elicited by the M. complanata extract. In some experiments, the endothelium was removed by in situ perfusion of the aorta with 1 mL of saline solution containing 0.2% desoxycholate immediately after the initial flushing with Krebs–Henseleit solution. The absence of endothelium was confirmed by obtaining a <5% relaxation in response to the addition of 1 µM acetylcholine. The influence of extracellular calcium on the vasoconstrictor effect elicited by the extract was analyzed using two different types of experiments. First, the susceptibility of this effect to the voltage-dependent Ca2+ channel blockers, nifedipine (1 µM) and verapamil (1 µM), was tested. In these experiments, blockers were allowed to act on the aortic rings for 5 min and afterwards, the extract was added to the organ bath. In other experiments, Ca2+-free Krebs–Henseleit solution was employed. The influence of intracellular calcium on the vasoconstrictor response produced by the extract was also studied on aortic segments whose intracellular calcium stores were depleted by a combined treatment with ryanodine (10 µM)/caffeine (10 mM).

2.2.2. Hemolysis assay

The hemolytic effects of the M. complanata extract and fractions were monitored according to the method previously described (Rottini et al., 1990) with some modifications. Briefly, samples for the assay contained a mixture (1 mL) of Alsever solution (120 mM dextrose, 30 mM sodium citrate, 7 mM NaCl, and 2 mM citric acid, adjusted to pH 7.4) with 50 µL of a 1% suspension of rat erythrocytes and the required volume of M. complanata extract or fractions. These samples were incubated at 37 °C for 30 min. After centrifugation (700 × g for 4 min at 4 °C), the A415 of the supernatant fluid containing the hemoglobin released from lysed erythrocytes...
was measured in a spectrophotometer (Lambda Bio, Perkin Elmer Co.). Each experiment was normalized with respect to complete hemolysis, which was measured by diluting the erythrocyte sample in deionized water instead of Alsever buffer. One hemolytic unit (HU50) was defined as the amount of protein sample required to cause 50% hemolysis.

Inhibition of hemolysis was evaluated after incubating the samples with cholesterol (50 or 100 μg/mL) in the Alsever solution for 30 min at 4 °C. In addition, hemolysis was determined after incubating the *M. complanata* extract (1.8 μg protein/mL) with 0.3, 1, or 3.3 mM p-bromophenacyl bromide (p-BPB) for 22 h at 4 °C. The influence of calcium and magnesium on the hemolytic effect elicited by the *M. complanata* extract (1.8 μg protein/mL) was determined by adding CaCl₂ or MgCl₂ (2.5, 5, 7.5, and 10 mM) to the Alsever buffer.

### 2.2.3. Determination of phospholipase A₂ activity

PLA₂ activity of the *M. complanata* extract was determined using a secretory PLA₂ colorimetric assay kit (Cayman Chemical, MI, USA). The assay uses the 1,2-dithio analogue of diheptanoyl phosphatidylcholine as substrate. Free thiols generated upon hydrolysis of the thiester bond at the sn-2 position by PLA₂ were detected using DTNB (5,5′-dithio-bis-(2-nitrobenzoic acid)). Color changes were monitored by a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories, CA, USA) at 414 nm, sampling every min for 10 min. PLA₂ activity was expressed as micromoles of hydrolyzed phosphatidylcholine per min per mg of protein.

### 2.2.4. Data analysis and statistics

Results are expressed as mean±S.E.M. from *n*=4 to 7 experiments. Concentration-response curves for *M. complanata* extract were plotted and fitted to the Boltzman equation, using the data analysis and graphics program Prism (GraphPad Software, San Diego, CA, USA). Multiple comparisons were made by a two-way ANOVA, followed by a post hoc Bonferroni test. In all cases statistical significance is indicated by *P*<0.05.

### 2.3. Reverse phase chromatography

Separation of the proteins contained in the *M. complanata* extract was carried out by reverse phase high performance liquid chromatography (RP-HPLC), using a C₄ semipreparative column (YMC-Pack, 10×250 mm; 5 μm particle diameter; 300 Å pore size; 2.5 mL/min) provided with a C4 guard column (YMC-Pack, 10×4.6 mm; 5 μm particle diameter; 300 Å pore size) and a filter (Alltech; 28689; 4 m, 2 μm particle diameter; 300 Å pore size) and a filter (Alltech; 28689; 4 m, 2 μm particle diameter; 300 Å pore size). HPLC solutions were: (A) 0.1% v/v trifluoroacetic acid (TFA) in water and (B) 0.09% v/v TFA in 90% v/v aqueous acetonitrile. A 5-mL sample-loading loop was used in all chromatographies, and the absorbance of the eluate was monitored at 206 nm.

### 2.4. Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as previously described (Schägger and Von-Jagow, 1987). Samples of 0.3–0.4 μg protein were run in 10–16% discontinuous gels at 90 V for 3 h at 4 °C, using Tris-glycine as buffer. Protein bands were visualized with both silver and Coomassie blue. Molecular masses were determined using broad-range polypeptide standards (Bio-Rad).

### 3. Materials

Phenylephrine, acetylcholine, nifedipine, verapamil, caffeine, ryanodine, ionomycin, cholesterol, and p-BPB were obtained from Sigma Aldrich (St. Louis, MO, USA). All salts, other reagents, and solvents were obtained from J.T. Baker (Phillipsburg, NJ, USA) or Sigma. Nifedipine and ionomycin were initially prepared as a stock solution in dimethyl sulfoxide (DMSO) and further diluted in deionized water. The highest concentration of DMSO used in the bioassay experiments was 0.2% v/v. Phenylephrine, acetylcholine, verapamil, and ryanodine were dissolved in deionized water. Ca²⁺-free Krebs–Henseleit solution was prepared by replacing CaCl₂ with MgCl₂ and 1.2 mM EDTA. Caffeine solution (10 mM) was prepared by dissolving the alkaloid in pre-warmed (37 °C) Krebs–Henseleit buffer before use, and the rat aorta was treated with caffeine by exchanging the bath solution with caffeine solution. Ionomycin, p-BPB, and cholesterol for the hemolytic assay were dissolved in ethanol. The highest concentration of ethanol used in the hemolytic assay experiments was 5%.

### 4. Results

#### 4.1. Effect of the *M. complanata* extract on isolated rat aortic rings

The *M. complanata* extract (0.1 to 316 μg protein/mL) produced a concentration-dependent vasoconstriction (EC₅₀=22.4±1.1 μg protein/mL) of the rat aortic rings, which was three-fold less than...
the effect of ionomycin (EC50 = 7.4 ± 1.1 μg protein/mL), used as a positive control (Fig. 1). The concentration-response curve (CRC) obtained for the extract did not show any significant difference when endothelium-denuded aortic rings were used, indicating that the M. complanata extract contains vasoconstrictor substances that directly interact with membrane proteins localized on the smooth muscle cells (Fig. 1). To determine whether these vasoactive substances were polypeptides, the extract (31.6 μg protein/mL) was incubated in a boiling water bath for 20 min. These denaturing conditions completely destroyed the vasoconstrictor effect of the extract (Fig. 2).

Fig. 2. Representative recordings showing the vasoconstrictor effect of the M. complanata extract (31.6 μg protein/mL) on rat isolated aortic rings under A) optimal and B) denaturing conditions.

Fig. 4. Concentration–response curves showing the hemolytic activity of the M. complanata extract and ionomycin on rat erythrocytes. Concentration represents protein content in the extract.

Fig. 5. Hemolytic activity of the M. complanata extract (1.8 μg protein/mL) after incubation with A) 50 and 100 μg/mL of cholesterol for 30 min at 4 °C and B) 0.33 and 3.30 mM of p-bromophenacyl bromide for 22 h at 4 °C. *p<0.05 versus control.

Fig. 3. Concentration-response curves showing the vasoconstrictor effect of the M. complanata extract on isolated aortic rings A) in presence of 1 μM verapamil and B) in a calcium-free medium or after incubation with 10 μM ryanodine/10 mM caffeine. *p<0.05 versus control.
To assess the contribution of extracellular calcium to the vasoconstriction elicited by the hydrocoral extract, its effect on the vascular tone was evaluated in the absence of extracellular Ca\(^{2+}\) and with either verapamil (1 μM) or nifedipine (1 μM), blockers of voltage-dependent Ca\(^{2+}\) channels. The results of these experiments showed that verapamil significantly reduced the contractile effect of the *M. complanata* extract only at a concentration of 100 μg protein/mL (Fig. 3A). A similar effect was obtained with nifedipine (data not shown). In the case of the experiments performed in the Ca\(^{2+}\)-free Krebs–Henseleit solution, the contractile response of the extract was significantly reduced in the range from 31.6 to 100 μg protein/mL. However, at a higher concentration (316 μg protein/mL) the vasoconstrictor effect was identical with and without Ca\(^{2+}\) (Fig. 3B).

The influence of intracellular Ca\(^{2+}\) on the aorta contraction elicited by the extract of *M. complanata* was examined by depleting Ca\(^{2+}\) stores of the sarcoplasmic reticulum with a combined ryanodine/caffeine treatment of the aortic rings (Rohra et al., 2003). This treatment resulted in a large rightward shift of the *M. complanata* extract CRC (Fig. 3B).

### 4.2. Hemolytic effect of the *M. complanata* extract

The *M. complanata* extract (0.2 to 10 μg protein/mL) showed hemolytic activity that was concentration-dependent.
(UH50=1.64±1.04 μg protein/mL) and was abolished after incubation in a boiling water bath for 20 min. The extract was approximately 47-fold more potent than ionomycin (UH50 of 77.8±1.03 μg/mL) (Fig. 4). The hemolytic effect of the extract (1.8 μg protein/mL) was significantly reduced after incubation with cholesterol (50 and 100 μg/mL) (Fig. 5A) or the PLA2 inhibitor p-BPB (0.3 and 3.3 mM) (Fig. 5B).

Considering that some phospholipases A2 require Ca2+ to achieve optimal enzymatic activity (Grotendorst and Hessinger, 2000; Nevalainen et al., 2004), the effect of divalent cations, Ca2+ and Mg2+, on the M. complanata extract-induced hemolysis was evaluated. Hemolytic activity was significantly increased in the presence of Ca2+ (1.25 to 10 mM) (Fig. 6). In contrast, Mg2+ increased hemolysis only at the highest concentration used (10 mM) (data not shown).

4.3. Phospholipase A2 activity

To confirm the presence of phospholipase A2 in the M. complanata extract, its PLA2 activity was determined using a secretory PLA2 colorimetric assay kit. The results obtained from this assay indicated that the hydrocoral extract had a PLA2 activity of 7.23±0.1 nmol min⁻¹ mg⁻¹, which was almost completely eliminated after incubation of the extract in denaturing conditions (0.033±0.003 nmol min⁻¹ mg⁻¹). PLA2 from bee venom, used as control, displayed an activity of 307.13±1.80 nmol min⁻¹ mg⁻¹.

4.4. Reverse phase chromatography and electrophoresis

To separate the toxins, RP-HPLC chromatography was employed. Nineteen fractions (F1-F19) were collected and evaluated by the isolated rat aorta and hemolytic assays (Fig. 7). Fractions F6, F8 and F9 (0.0286 μg protein/mL) induced a transitory vasoconstrictor effect, while F11 showed a sustained vasoconstriction (0.057 μg protein/mL) of aortic rings (Fig. 8). On the other hand, determination of the hemolytic activity of each fraction (0.2 μg protein/mL), except for F6 and F9 (insufficient quantity), showed that only F4 produced significant hemolysis. Fractions collected after F11 had neither hemolytic nor vasoconstrictor activity.

Electrophoretic analysis of fractions F4, F8, and F11 revealed that F4 contained a prominent protein band with a molecular mass of approximately 30 kDa, while F8 and F11 contained prominent protein bands of 15 and 20 kDa, respectively (Fig. 9).

5. Discussion

Millepora species are widely known because they can cause painful stings in humans after contact. It has been reported that the nematocysts of some of these cnidarians mainly contain cytolytic proteins (Radwan, 2002; Radwan and Aboul-Dahab, 2004). In the present study, it was found that the M. complanata extract produces contraction of isolated rat aortic rings and hemolysis. Both activities were abolished after incubation in a boiling water bath for 20 min, indicating that the toxins responsible for these effects are heat labile. These results are in accordance with other studies showing that cnidian toxins are proteins with biological activities labile to temperatures above 60 °C (Watters and Yanagihara, 2003).

The M. complanata extract, which contains a complex mixture of components, was only five-fold less potent than ionomycin in inducing vasoconstriction. The CRC obtained for the effect of the extract on endothelium-denuded aortic rings did not show any significant difference from the curve obtained when intact aortic rings were used, indicating that the extract contains vasoconstrictor substances that act directly on vascular smooth muscle cells rather than on endothelial cells.

Vascular smooth muscle contraction is activated by a rise in intracellular Ca2+ concentration ([Ca2+]i). Increases of [Ca2+]i may result from calcium influx across the plasma membrane or Ca2+ release from the sarcoplasmic reticulum (SR) (Tognarini and Moulds, 1997). In this study, we carried out a series of experiments to investigate the role of extracellular and intracellular Ca2+ on the vasoconstrictor effect induced by the extract of M. complanata.

First, the vasoconstrictor effect of the extract was evaluated in the presence of nifedipine and verapamil. In these experiments, the contractile response of the M. complanata extract was not significantly affected by either nifedipine or verapamil, suggesting that the influx of Ca2+ through L-type voltage-dependent Ca2+ channels does not participate prominently in the vasoconstrictor effect elicited by the extract. On the other hand, when extracellular calcium was absent, the contractile response of the extract was significantly reduced, but not completely abolished. These findings suggest that extracellular calcium plays an important role in the vasoconstrictor effect of the M. complanata extract. This Ca2+ might be entering into the smooth cells through T-type voltage-dependent Ca2+ channels (McDonald et al., 1994; Bolotina, 2000) and receptor-operated, nonselective cation channels (Fasolato et al., 1994; Bolotina, 2000). Considering that the extract-induced vasoconstriction did not completely disappear in the absence of extracellular Ca2+, it is likely that this effect might involve the release of Ca2+ from intracellular stores.

The SR is an important calcium reservoir in vascular smooth muscle cells. Release of calcium from the SR results from activation of either the ryanodine receptors (RyRs) or the 1,4,5-inositol triphosphate (IP3) receptors (IP3R). The function of the SR as a calcium reservoir can be blocked via a ryanodine/caffeine combined treatment, which prevents refilling of the SR because the ryanodine receptors remain open (Rohra et al., 2003). In this study, we observed that depletion of the SR induced a rightward shift of the CRC of the M. complanata extract, which supports our
hypothesis that *M. complanata* extract induces vasoconstriction not only by increasing the influx of extracellular calcium, but also by provoking the release of calcium from the SR, possibly from the ryanodine-sensitive store.

It has been reported that a sustained vasoconstriction is mainly due to an influx of extracellular calcium, and a transitory vasoconstriction is mainly due to calcium release from the SR (Rohra et al., 2003). In the pharmacological evaluation of the chromatographic fractions obtained from the *M. complanata* extract, we observed that F6, F8, and F9 produced a transitory vasoconstrictor effect, whereas F11 induced a sustained vasoconstriction. SDS-PAGE of F8 and F11 showed the presence of 15- and 20-kDa proteins, respectively. These findings suggest that the hydrocoral extract contains vasoconstrictor toxins with different mechanisms of action that involve both increased extracellular Ca\(^{2+}\) influx and increased Ca\(^{2+}\) release from the SR.

We found that the *M. complanata* extract also caused a concentration-dependent hemolytic effect on rat erythrocytes. It has been proposed that cnidian hemolysins must interact with erythrocyte membrane lipids to produce hemolysis, since pre-incubation with certain membrane lipids blocks the hemolytic effects of these toxins. In the present study we found that the hemolysis produced by the *M. complanata* extract is reduced in the presence of cholesterol. It is worth noting that the hemolytic activity of the extracts obtained from other fire corals (*M. dichotoma* and *M. platyphylla*) is most effectively inhibited by dihydrocholesterol (Radwan et al., 2002; Radwan, 2002). In contrast, hemolysis induced by the extracts obtained from other cnidarians, like the jellyfishes of the genus *Aurelia* and *Cassiopea*, is inhibited after incubation with phosphatidylcholine (Radwan et al., 2001).

It has been previously described that the hemolytic activity elicited by *M. platyphylla* is mainly caused by Milleporin-1, a new phospholipase A\(_2\) (PLA\(_2\)) which is a 32.5 kDa protein detected in the crude extract of the hydrocoral (Radwan and Aboul-Dahab, 2004). Consistent with these findings, we found that the hemolytic activity of the *M. complanata* extract was significantly reduced after incubation with p-PPB, an inhibitor of PLA\(_2\) activity. This inhibitor interacts with His-48 at the substrate binding site of the PLA\(_2\) enzyme and induces chemical modification, which inactivates the enzyme (Kini, 2005; Kuruppu et al., 2005). Inhibition of the *M. complanata*-induced hemolysis by p-PPB indicates that this species produces hemolysins with PLA\(_2\) activity. Similar proteins have been found in most venomous animals, including snakes, bees, scorpions, and cnidian species (Nevalainen et al., 2004). Most of these animal venom enzymes are toxic and induce a wide spectrum of pharmacological effects, which include neurotoxic, cardiotoxic, myotoxic, hemolytic, convulsive, anticoagulant, and other effects (Kini, 2005). Some PLA\(_2\) proteins require divalent cations for maximal toxic activity. For example, β-PLA\(_2\) obtained from the anemone *Aiptasia pallida* is more efficient in the presence of divalent cations, especially calcium (Grotendorst and Hessinger, 2000). Similarly, we found that the hemolytic activity of the *M. complanata* extract increased in the presence of higher Ca\(^{2+}\) concentrations. Magnesium also increased the hemolytic activity of the extract, but only at the highest concentration tested (10 mM). Finally, we confirmed that the *M. complanata* extract contained PLA\(_2\) proteins by directly measuring its phospholipase activity, which was approximately 42-fold less potent than that of a PLA\(_2\) from bee venom.

Evaluation of the hemolytic activity of the chromatographic fractions obtained from the *M. complanata* extract indicated that only F4 had significant hemolytic activity. SDS-PAGE of this fraction showed a band with a molecular mass of approximately 30 kDa, which is similar to that of milleporin-1 (32.5 kDa). Thus, the hemolytic effect elicited by the *M. complanata* extract is mainly caused by a hemolysin with PLA\(_2\) activity and mass similar to Milleporin-1 obtained from *M. platyphylla*.

In conclusion, this study showed that the *M. complanata* crude extract displays two different biological activities: vasoconstriction of isolated rat aortic rings and hemolysis of rat erythrocytes. Vasoconstriction is caused primarily by a 15 kDa protein that induces a transitory contraction related to calcium release from the SR, and a 20 kDa protein that elicits a sustained contraction, probably associated with increased influx of extracellular calcium into the vascular smooth muscle cells. In addition, the hydrocoral extract displays PLA\(_2\) activity, and it contains a 30 kDa hemolysin similar to Milleporin-1, a PLA\(_2\) previously detected in *M. platyphylla* venom.

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