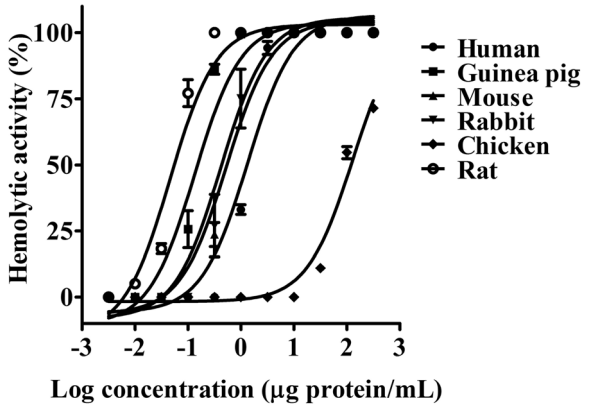

Fig. 1 Concentration-response curves showing the hemolytic activity of the *M. alcicornis* aqueous extract on erythrocytes from various species

inactivated at temperatures higher than 60 °C, while at preincubation temperatures lower than 45 °C, the hemolytic activity of the extract was conserved (Fig. 2c).

The hemolysis caused by the hydrocoral extract was assessed in the presence of divalent cations such as Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, or Ba^{2+}. These experiments showed that the hemolytic activity was depressed by Cu^{2+} 0.1 mM or Zn^{2+} 6 mM, however it was increased by addition of Ca^{2+} and Mg^{2+}. In the case of Ba^{2+}, concentrations lower than 6 mM increased the hemolytic activity of the extract, whereas concentrations higher than 8 mM induced inhibition of this activity (Table 2). Hemoysis produced by the extract was reduced after incubation with EDTA 0.34 mM and sharply disappeared at EDTA 0.43 mM (Table 3).

The hemolytic activity of the *M. alcicornis* aqueous extract was significantly reduced after incubation with the PLA_2 inhibitor *p*-BPB. In these experiments, the concentration response curves were rightward shifted depending on the concentration of the inhibitor. In the absence of *p*-BPB the HU_{50} was 0.069 ± 0.081 μg protein/mL, after incubation with 0.33 mM *p*-BPB the HU_{50} was 7.42 ± 0.13 μg protein/mL, with 1.0 mM *p*-BPB the HU_{50} was 6.51 ± 0.14 μg protein/mL. Finally, at 3.3 mM *p*-BPB a greater inhibitory effect was observed and the value of the HU_{50} increased up to 35.89 ± 0.16 μg protein/mL (Fig. 3).

Table 1 Hemolytic activity of the *M. alcicornis* extract on several types of erythrocytes

Erythrocytes type	Hemolytic unit (HU_{50})
Rat	0.042 ± 0.005
Guinea pig	0.29 ± 0.15
Mouse	0.41 ± 0.015
Rabbit	0.63 ± 0.35
Human	1.0 ± 0.31
Chicken	129 ± 8.2*

*$p < 0.05$ versus hemolysis on rat erythrocytes

SDS-PAGE electrophoresis and zymography

The *M. alcicornis* aqueous extract contains proteins with a broad range of molecular weights, between 8 and 200 kDa and under reducing conditions, the electrophoretic profile was altered (Fig. 4a). Two main hemolytic zones were identified by zymography, one of which corresponded to a band of approximately 28–30 kDa, and the other one was related to a broad band of 200 kDa, approximately (Fig. 4b). Under reducing conditions, the 200 kDa band disappeared, which suggested that it consists of two or more dimers. The zymography analysis showed that the 28 kDa band possessed PLA_2 activity, while the 200 kDa did not elicit this enzymatic activity (Fig. 4c).

Lethality assay in mice and systemic effects

When intravenously injected, the aqueous extract of *M. alcicornis* was lethal to mice with a LD_{50} of 17 μg protein/g of body weight (Table 4). At doses lower than the LD_{50} (1.5 and 3.0 μg protein/g) the extract induced forced respiration immediately after the administration, but mice recovered within a few minutes. Mice also showed a progressive depressed responsiveness, which was usually pain related. However, they progressively recovered their normal behavior. At higher doses (6 and 12 μg protein/g), the aqueous extract also induced respiratory difficulty, hypoactivity, and other symptoms such as grooming, paralysis of the hind legs, intestinal inflammation, hemoglobinuria, and after a while, complete paralysis. In some cases, respiratory failure and convulsions preceded death. Doses higher than the LD_{50} (24 and 48 μg protein/g) induced symptoms similar to those of the lower doses, although in most cases, deaths occurred in a shorter time. The denatured aqueous extract of *M. alcicornis* did not have any effect in mice.

Histopathological analysis

LM images showed histopathological changes in kidney, lung and liver tissues but not in heart, brain or skeletal muscle. In kidney sections, glomerular capillaries appeared congested with proteinaceous material within the tubules. In some cases, acute tubular necrosis (Fig. 5a and b) was observed. Lung sections showed severe alveolar epithelial cells degeneration and desquamation, damage to capillaries, and marked infiltration of erythrocytes and proteinaceous material within alveoli (Fig. 5c and d). Liver sections showed varying degrees of pathological lesions, with acute vascular congestion and areas