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In vitro pharmacological activities of the extracts from red ant *Formica aquilonia* as potential therapeutic agents

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Abstract

The extracts of Chinese red ant *Formica aquilonia* were screened for cytotoxicity, antioxidation and anti-inflammatory properties, prolyl oligopeptidase (POP) inhibition, and the effect on the catecholamine secretion. Dried ants were successively extracted with hexane, ethyl acetate, aqueous acetone and distilled water. Both the hexane and ethyl acetate extracts exhibited a high level of POP inhibitory activity; ethyl acetate extract was also significantly cytotoxic against the rat liver cancer cells. In the assay with anti-inflammatory assay, the residue of hexane extract suppressed weakly COX-2 activity. Inhibition of the acetylcholine-dependent catecholamine secretion from the bovine adrenal chromaffin cells was most pronounced with the ethyl acetate extract. The aqueous acetone extract exhibited antioxidant activity in a diphenylpierylhydrazyl assay. These results indicate that *F. aquilonia* contains promising therapeutic agents.

Key words red ant extract, cytotoxicity, anti-inflammation, POP, catecholamine secretion, antioxidant.

Introduction

Pharmacological studies on entomoresouce are largely based on traditional medicine in Asia and the Central and South America.[1,2] Ants have been widely used and very highly regarded in China as a tonic for over 3,000 years. The ants are believed to affect blood circulation and metabolism, bolster the immune system, reduce inflammations and pain, palliate asthma and rheumatoid arthritis, retard aging, and exhibit other pharmacological effects.[3-9] The species primarily used as food and medicine in China is the weaver ant, *Polyrhachis vicina*, a relatively large and black creature, which is widely distributed in southern China. Recent studies on the ant of *P. vicina* demonstrated great medical potential of insects and opened way to the development of pharmacological agents from the compounds of insect origin.[10-12] The related Chinese medicinal ant *P. lamellidens* has also been used for many years clinically as well as rustic medicine to treat rheumatoid arthritis and hepatitis. It has recently been reported that the ethanol extract from *P. lamellidens* has remarkable analgetic and anti-inflammatory activities.[13] Two novel aliphatic polyketide lactones were isolated from the ether fraction of the methanolic extract of *P. lamellidens*,[14] but their biological activities remain to be analyzed.

In contrast to the *Polyrhachis* species, there is little information on the pharmacological activities of the red ant *Formica aquilonia*, which is also widely used in

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folksy disease cures.[15] In the present paper, we describe cytotoxic, antioxidant, anti-inflammatory, prolyl oligopeptidase-suppressive and catecholamine secretion-inhibitory effects of *F. aquilonia* extracts.

Materials and Methods

Insect materials: *Formica aquilonia* red ants were harvested at an altitude of 800~1,100 m in the Changbai Mountains, Linjiang, China by the Jilin Province Lisheng Source Biological Preparations Limited Company. They were suffocated, air-dried and stored at -80℃ until use.

Stepwise extraction: A sample of 153 g dry ants was milled in a blender (WB-I, Osaka Chemical) and the powder was successively extracted with hexane (500 ml, twice), ethyl acetate (500 ml, twice), aqueous acetone (acetone/water = 7/3, 500 ml, twice), and distilled water (500 ml, twice). The extraction was done at ambient temperature for 24 h with each solvent. The extracts of hexane and ethyl acetate were subsequently centrifuged at 5,000 rpm for 5 min and yielded 27.76 g of hexane supernatant (s), 4.69 g hexane residue (r), 1.36 g ethyl acetate supernatant (s) and 1.95 g ethyl acetate residue (r). A residue of 25.40 g was obtained after evaporation of the aqueous extract, and 21.20 g after evaporation of the water extract. All six preparations were taken for the following assays.

Cytotoxic assay: Rat hepatoma cells (dRLh84) were used to assess cytotoxicity. The cells were trypsinized, diluted with Dulbecco's modified Eagles medium (DMEM) containing 10% new-born bovine serum, 4 mM glutamine, and a mix of antibiotics (a mixturte type of 50 U/ml penicillin and 50 μg/ml streptomycin- ICN Biomedicals, and 100 μg/ml kanamycin- Sigma), and further kept at 37℃ in 5% CO_2 under humidified conditions. For the tests of the hexane and ethyl acetate extracts, 200 μl of the cell suspension containing 2.5 × 10^4 cells/ml were dispensed per well of a 96-well microplate. The plate was pre-incubated for 24 h, extract aliquots were added in 1 μl dimethylsulfoxide (DMSO) (controls received DMSO alone), and the cell survival rate was measured by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide (MTT) assay after 48 h.[16] In the case of the water-soluble samples (aqueous acetone and water extracts), 100 μl of the cell suspension at a density of 5 × 10^4 cells/ml were used and extract samples were added in 11 μl of phosphate-buffer saline (PBS) (controls were treated with PBS alone).

POP inhibition assay: Prolyl oligopeptidase (POP) inhibition activity was measured by the modified method of Yoshimoto and Walter.[17] The reaction mixture consisted of 50 μl of the 2 mM benzyloxycarbonyl-glycyl-*l*-prolyl-*p*-nitroamilide (Z-Gly-Pro-*p*NA, Seikagaku) in 40% 1,4-dioxane and 10 μl of the sample in 890 μl 0.1 M Tris-HCl buffer (pH 7.0). After a pre-incubation at 37 ℃ for 10 min, the reaction was started by the addition of 50 μl POP solution (0.1 U/ml, POP of *Flavobacterium meningosepticum* purchased from Seikagaku). The amount of released *p*-nitroaniline released after 30 min incubation at 37℃ was determined colorimetrically at 410 nm with UV-visible spectrophotometer (Shimadzu UV-1600) (A_s). A_{410} of the reaction mixture containing 990 μl of buffer and 10 μl of the sample was separately measured in the same manner (A_{sb}). A control was made by the addition of 990 μl of buffer instead of the sample (A_c). POP inhibitory activity was calculated as follows: POP inhibitory activity (%) = $[1 - (A_s - A_{sb}) / A_c] \times 100$. Benzyloxycarbonyl-L-prolyl-prolinal (Z-Pro-prolinal, Funakoshi) was used as a positive control.

DPPH radical scavenging assay: Colorimetry with 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used to assess the free radical-scavenging activity.[18] The reaction mixture consisted of 1 ml of 0.5 mM DPPH solution (dissolved in ethanol) and 200 μl of the sample in 800 μl 0.1 M Tris-HCl buffer (pH 7.4). The mixture was incubated at ambient temperature for 20 min, and absorbance was read at 517 nm. Ascorbic acid was as a positive control. Inhibitory activity of the DPPH radical was calculated as follows: DPPH radical scavenging activity (%) = $(1 - (A_s - A_{sb}) / A_c) \times 100$ (A_s, absorbance of test sample solution; A_{sb}, absorbance of blank sample; A_c, absorbance of positive control).

COX-1, COX-2 inhibitory assay: Cyclooxygenase (COX) inhibition was assessed calorimetrically according to the supplier's instructions supplied with

colorimetric COX (ovine) Inhibitor Screening Assay Kit (Cayman Chemical, No.760111). The reaction mixture was consisted of 10 μl of an ant extract and 10μl of Heme in 150 μl 100 mM Tris-HCl (pH 8.0) containing 10 μl of either COX-1 or COX-2 solution. After a pre-incubation for 5 min at 25℃, the reaction was started by the addition of colorimetric substrate (20 μl) and arachidonic acid (20 μl). The degree of COX-1 or COX-2 inhibiton was determined after 5 min incubation at 25 ℃ by measuring A_{590} with a microplate reader. The inhibitory activity was calculated in % with the following formula: $(1 - (A_s - A_{sb}) / A_c) \times 100$ (A_s, absorbance of test sample solution; A_{sb}, absorbance of the sample blank; A_c, absorbance of control). NS398 (COX-PAK, Cayman) was used as a positive control.

Catecholamine secretion and Ca^{2+} influx in chromaffin cells: Bovine adrenal glands were kindly provided by the Center of Iwate Chikusan Center and the chromaffin cells were prepared by collagenase digestion as described previously.[19] The isolated cells were suspended in Eagle's minimum essential medium supplemented with 10% calf serum and antibiotics (100 U/ml of penicillin, 100 μg/ml of streptomycin and 0.3 μg/ml of amphotericin B). The cells were plated at a density of 7.5×10^5 cells/well in a 12-well plate, and were cultured at 37℃ in a 5% CO_2 incubator. After 4 days of culturing, the cells were washed twice with the Krebs-Ringer-HEPES (KRH) buffer (pH 7.4) and then pre-incubated with or without the test agents in KRH buffer for 10 min at 37℃, and incubated with or without 50 μM acetylcholine (ACh) for 7 min. The catecholamines secreted into the medium were extracted with 0.4 M perchloric acid, absorbed on aluminum hydroxide, and quantified by the ethylenediamine condensation method,[20] using a fluorescence spectrophotometer (650-10S, Hitachi). ^{45}Ca was used to measure calcium influx. After a pre-incubation, the cells were incubated with or without 100 μM ACh in the KRH buffer containing 123 kBq/ml 45Ca for 7 min in the presence or absence of the samples. The reaction was stopped on ice and the cells were washed twice with cold Ca^{2+}-free KRH buffer. The cells were lysed with 10% Triton X-100 and the radioactivity was measured by a liquid scintillation counter (LSC-6100, Aloka).

Results

Cytotoxic effects on rat hepatoma cells: All six extract samples prepared from the red ants were tested on the proliferation of rat hepatoma cells. As shown in Fig. 1, the extracts in hexane and ethyl acetate showed significant cytotoxic effect in a dose-dependent manner. At concentrations of 500 μg/ml, both the supernatants and the sediments of these extracts fully suppressed the cell growth and caused necrotic cell death. The residues of ethyl acetate extracts with ID_{50} value 134.6±9.5 μg/ml was most effective; the supernatant of hexane extracts with ID_{50} of 359.5 ± 8.2 μg/ml, the residue of hexane extracts with ID_{50} of 417.0±14.0 μg/ml, and the supernatant of ethyl acetate extracts with ID_{50} of 439.9 ±

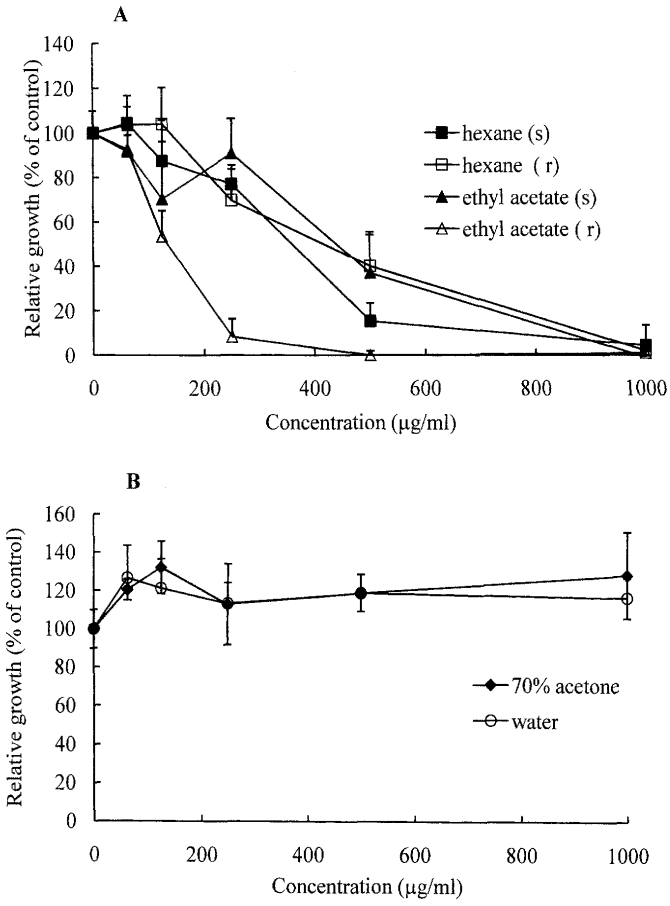

Fig. 1. Dose-dependent cytotoxic effects of the red ant extract on the rat hepatoma cells (dRLh84).
dRLh84 cells were cultured with various concentrations of extracts for 48 h and the cell cytotoxic effect was evaluated by the MTT assay. (A), hexane and ethyl acetate extracts; (B), 70% acetone and water extracts. The extracts of hexane and ethyl acetate were centrifuged and yielded hexane supernatant (s), hexane residue (r), ethyl acetate supernatant (s), and ethyl acetate residue (r) as shown in the Materials and Methods. These also apply to Figs. 2, 3, 4 and 5. Each data represents the mean±S.D. of n=3.

16.5 μg/ml (Fig. 1A). The solvent (0.5% DMSO) had no effect. No cytotoxic effect was also found in the extracts prepared with aqueous acetone and distilled water, respectively (Fig. 1B).

Effects on DPPH radical scavenging activity: DPPH was used to assess the effects of extracts on the scavenging of oxidative radicals. At a dose of 1 mg/ml only the supernatant component of the hexane was inactive. All other extracts showed significant antioxidant activities. The most efficient acetone extract had about 70% of the antioxidant potential of ascorbic acid that was used as positive control (Fig. 2).

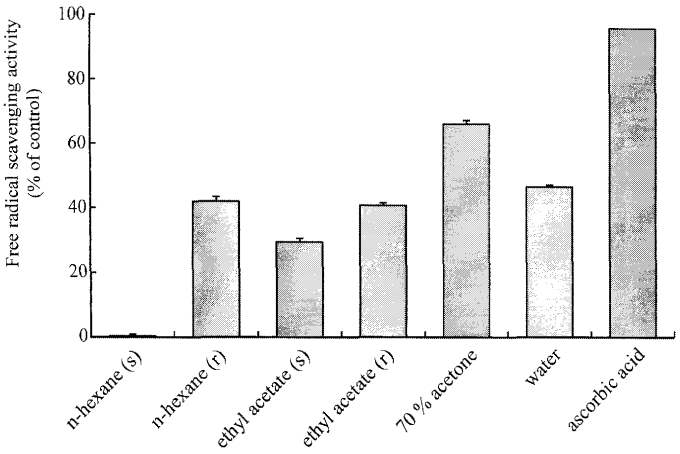

Fig. 2. Effects of the ant extracts on DPPH scavenging activity. The reaction mixture consisted of 1 ml of 0.5 mM DPPH radical solution and 0.8 ml of the ant extracts (1 mg/ml), or ascorbic acid as a positive control (0.1 mg/ml). The solution was rapidly mixed and scavenging capacity was measured at 517 nm. Data (n=3) are expressed as mean± S.D. of the DPPH decrease in comparison with control (100%).

Inhibition of the COX-1 and COX-2 activities: COX-1 is constitutively expressed while COX-2 is mitogen-inducible. In general, targeting COX-2 and a highly selective COX-2 inhibition have been considered as anti-inflammatory effects of the non-steroidal anti-inflammatory drugs (NSAIDs),[21] and this present study used a positive agents, NS398, which is selective COX-2 inhibition (Fig. 3). At a dose of 100 μg/ml, COX-2 activity was inhibited by the supernatant component of the hexane extract and by both the supernatant and the residue of ethyl acetate extract, but only the hexane supernatant exhibited a significant COX-2 reduction (28%) without suppressing the COX-1 activity (Fig. 3). Similar to the cytotoxic activity, the extracts of aqueous acetone and distilled water had no COX-2 inhibitory effects.

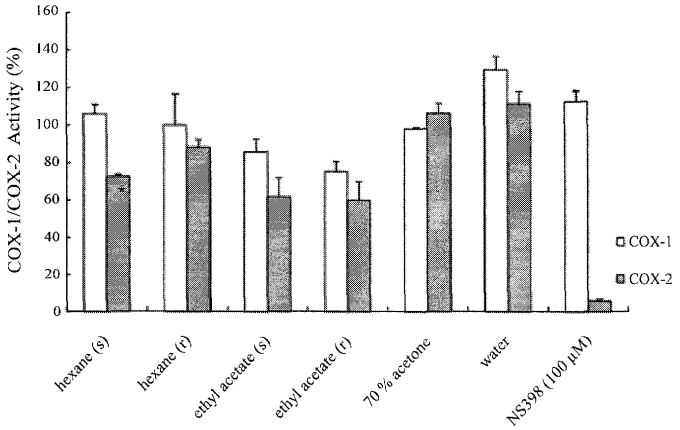

Fig. 3. Effects of the ant extracts on the COX-1 and COX-2 inhibitory activities.
COX-1 and COX-2 inhibitory activities were evaluated by measuring A_{590} with a microplate reader. Results were calculated by comparison with the control activity, assumed to be 100%. NS398 was used as a positive control. Each data represents the mean± S.D. of n=3. *p<0.01 compared with the control.

Inhibition of the POP activity: Evaluation of POP inhibitory activity is used in studies of the cerebral functions of mammals.[22-24] All 6 ant extracts were investigated in respect to POP inhibition. No influence on POP activity was detected with the residues of the hexane and ethyl acetate extracts (data not shown) and with the extracts prepared with aqueous acetone and distilled water (Fig. 4). However, the supernatant fraction of the hexane and ethyl acetate extracts exerted dose-dependent inhibitory effects with ID_{50} of 27.1 ± 5.3 μg/ml and 40.3 ± 0.1 μg/ml, respectively (Fig. 4).

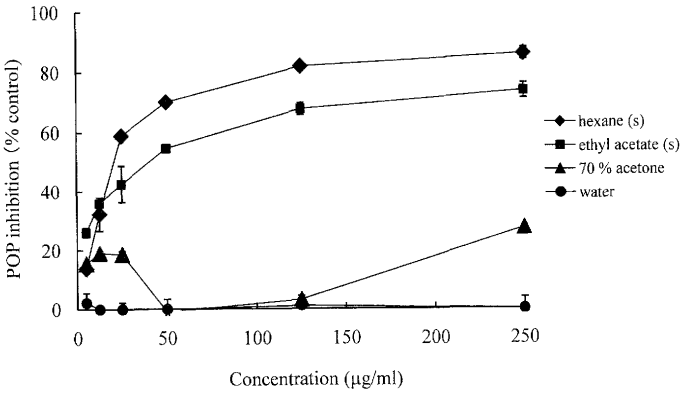

Fig. 4. Effects of the ant extracts on POP inhibitory activity. The reaction mixture was incubated with various concentrations of the ant extracts (the supernatants of hexane and ethyl acetate extracts; aqueous acetone and water extracts) at 37℃ for 30 min and the reaction was stopped on ice. Each data represents the mean± S.D. of n=3.

Effects on catecholamine secretion activity in chromaffin cells of bovine adrenal glands: The adrenal chromaffin cells are widely used as a model of sympathetic neurons in the studies of catecholamine secretion.[25] We examined the effects of ant extracts on the catecholamine secretion from cultured bovine adrenal chromaffin cells stimulated by ACh. As shown in Fig. 5, the supernatant fraction of the hexane extract and both fractions of the ethyl acetate extract decreased catecholamine secretion significantly at dose of 0.5 mg/ml. The extracts in aqueous acetone and distilled water were inactive. In the case of the ethyl acetate extract, the effects of different doses were examined and we found that the extract inhibited the ACh-induced secretion of catecholamines in a concentration-dependent manner (ID_{50} value of 155.6 ± 5.8 μg/ml) (Fig. 6A). Ca^{2+} influx into the chromaffin cells is essential for the catecholamine secretion stimulated by ACh. Next, therefore, the effect of the extract on Ca^{2+} influx was investigated. The ethyl acetate extract reduced the ACh-induced Ca^{2+} influx. The concentration-response curve of the Ca^{2+} influx inhibition with ID_{50} value of 160.7 ± 3.6 μg/ml was similar to that of the reduction of catecholamine secretion (Fig. 6B).

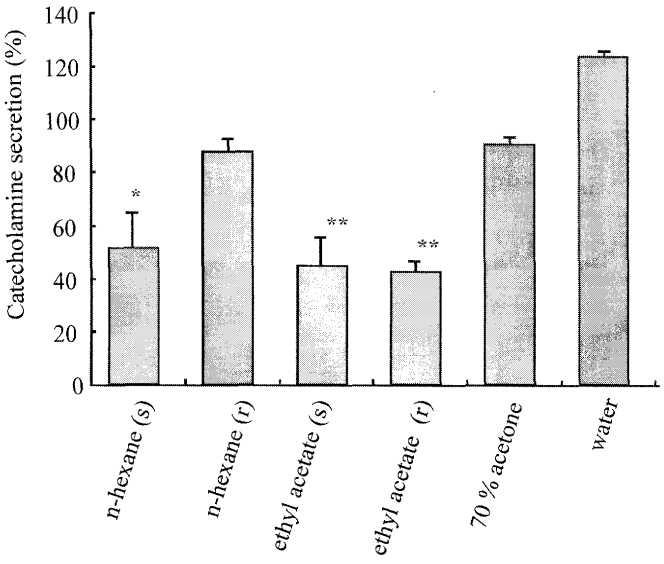

Fig. 5. Effects of the ant extracts on catecholamine secretion from the ACh-stimulated bovine adrenal chromaffin cells.
Cultured chromaffin cells were pre-incubated in the presence or absence of the ant extracts (0.5 mg/ml) in KRH buffer at 37 ℃ for 15min, and then incubated with the same extract in the presence or absence of ACh (50 μM) for 7 min. ACh-evoked secretion was assigned as 100% value. Values are means ± S.D. of n=3. *p<0.05, **p<0.01 compared with the ACh-evoked secretion.

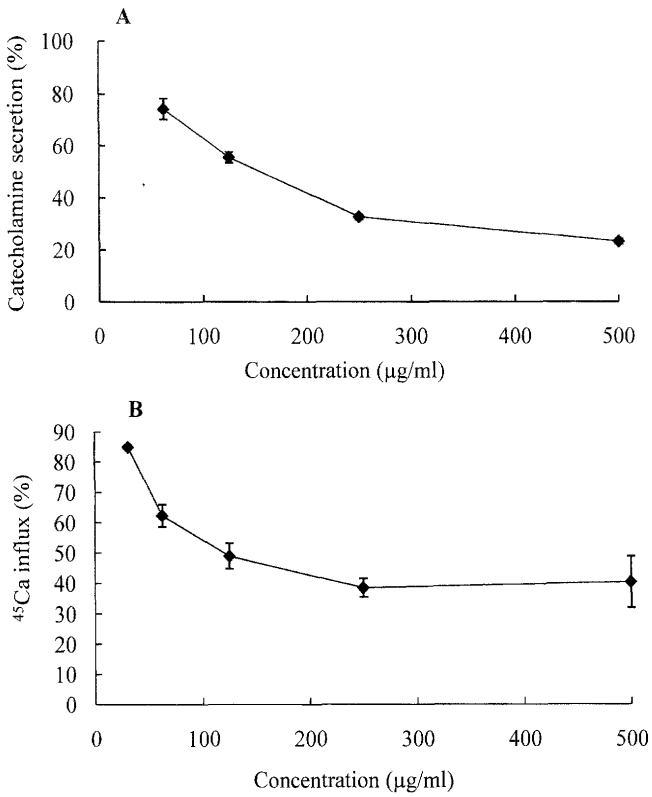

Fig. 6. Effects of the residue (sediment after centrifugation) of ethyl acetate extract on the catecholamine secretion (A) and Ca^{2+} influx (B) in bovine adrenal chromaffin cells.
The cultured chromaffin cells were pre-incubated in the presence or absence of the residue of ethyl acetate extract residue (0.5 mg/ml) in KRH buffer at 37℃ for 15 min, and then incubated with the same extract in the presence or absence of ACh (50 μM) for 7 min. ACh-evoked secretion was assigned as 100% value. Values are means ± S.D. of n=3.

Discussion

The use of ants in Chinese traditional medicine stimulated pharmacological research that revealed anti-inflammatory and analgestic properties of the black ants *Polyrhachis lamellidens* and *P. vicina*.[9,13] Three animal models were chosen for the screening of anti-inflammatory effects: xylene-induced ear edema, acetic acid-induced peritoneal capillary permeability, and leucocytes emigration. The results suggested that the anti-inflammatory agents occurred mainly in the water fraction of the ethanol extract of these species.

The red ant *Formica aquilonia* is known as a forest-dwelling animal[26] and has also been widely used in the folk medicine but search for effective compounds has hitherto been limited to isolation of the defensin gene[27] and identification of chemical substances such as cAMP, cGMP, ferritin, SOD *etc.*[15] Our *in vitro* tests of

cytotoxic, antioxidant, and anti-inflammatory activities and of the POP and effect on catecholamine secretion represent the first attempt to prove existence of pharmacologically active compounds in the ant in question. The results demonstrate that both the residue and supernatant fractions of the ethyl acetate extract display remarkable cytotoxic effect on the rat hepatoma cells and inhibitory-effect on the ACh -stimulated Ca^{2+} influx and consequent catecholamine secretion in the chromaffin cells of bovine adrenal glands (Figs. 1A, 5, 6). This observation is consistent with the folkloric use of this ant species to treat tumors and vascular disorders.

The supernatant fraction of the hexane extract showed favorable activities in the *in vitro* COX-1/COX-2 inhibitory assay (Fig. 3) and the prolyl oligopeptidase (POP) inhibitory assay (Fig. 4). The inhibition of POP-mediated conversion of unsaturated fatty acid has been proposed as a treatment in preventing memory loss, for example in the Alzheimer's disease.[28] It should be noted that NMR data of the crude hexane extract indicated the presence of unsaturated fatty acids as a major component (data not shown), but their chemical structures remain to be analyzed. It is not excluded that extracts from other social Hymenoptera also contain compounds exerting favorable effects on nervous functions. We found that the extracts from the *Vespa simillima* wasp enhanced long-term memory and partly alleviated MK-801-induced learning disability in mice.[29] Investigations of the effects of hexane and ethyl acetate extracts on the cerebral functions of mammals are in progress.

Selective COX-2 inhibitors are being developed for the treatment of rheumatic diseases. Some COX-2 inhibition are also considered for mitigation of the Alzheimer's disease symptoms.[30] Although the present study provides no direct evidence for anti-inflammatory activity of the red ant extract, the studies on *P. lamellidens*[13] and *P. vicina*[9] suggest that the POP inhibition is indicative of anti-inflammatory extract effects. However, only the water fraction of the ethanol extract of *P. lamellidens* had remarkable anti-inflammatory effect in animal models, whereas high level of POP inhibitory activity in *F. aquilonia* was found in the supernatant fraction of the hexane and ethyl acetate extracts. Biologically active components of these fractions will be isolated and their structures determined.

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