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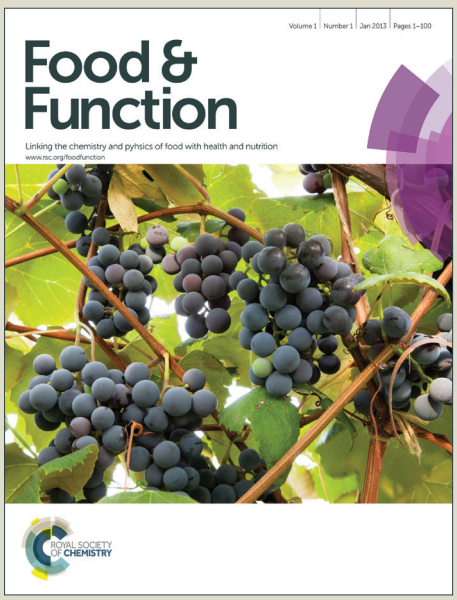

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Water-soluble jack-knife prawn extract inhibits 5-hydroxytryptamine-induced vasoconstriction and platelet aggregation in humans

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Runnning title

Inhibitory effect of jack-knife prawn extract on 5-HT-related vasospastic diseases

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Abstract

Coronary artery spasm plays an important role in the pathogenesis of various ischemic heart diseases or serious arrhythmia. The aim of this study is to look for functional foods which have physiologically active substances preventing 5-hydroxytryptamine (5-HT)-related vasospastic diseases including peri- and postoperative ischemic complications of coronary artery bypass grafting (CABG) from ocean resources in Japanese coastal waters. First, we evaluated the effect of water-soluble ocean resource extracts on the response to 5-HT in HEK293 cells which have forcibly expressed cyan fluorescent protein-fused 5-HT_{2A} receptors (5-HT_{2A}-CFP). Among 5 different water-soluble extracts of ocean resources, the crude water-soluble jack-knife prawn extract (WJPE) significantly reduced maximal Ca^{2+} influx induced by 0.1 μM 5-HT in a concentration-dependent manner. The Crude WJPE significantly inhibited, in a concentration-dependent manner, 5-HT-induced constriction of human saphenous vein. 5-HT released from activated platelets plays a crucial roles in the constriction of coronary artery. Next the WJPE was purified for applying the experiment of 5-HT-induced human platelet aggregation. The purified WJPE significantly inhibited 5-HT-induced human platelet aggregation also in a concentration-dependent manner. Based on our findings, jack-knife prawn could be one of a functional food with health-promoting benefits for most people with vasospastic diseases including patients who have gone CABG.

Keywords

jack-knife prawn, human, saphenous vein, serotonin-induced vasoconstriction, serotonin-induced platelet aggregation

Introduction

Coronary artery disease (CAD) is a lifestyle-related disease and the leading cause of mortality in Western countries. In Japan, the prevalence rate of CAD is increasing with the westernization of dietary habits and sedentary lifestyle.[1] Coronary artery bypass grafting (CABG) is an effective treatment for severe CAD. The saphenous vein (SV) continues to be the most commonly used conduit vessel for CABG because of its ready availability and suppleness[2]; however, approximately half of saphenous vein grafts (SVG) eventually develop high-grade stenosis or obstruction, called SVG disease, within several years after operation.[3] SVG disease have a critical impact on the life prognosis of patients who have undergone CABG. Coronary artery spasm plays an important role in the pathogenesis of various ischemic heart diseases or serious arrhythmia.[4] We have investigated the mechanisms of vasospasm to be aimed at long-term patency of bypass grafts.[5, 6] We think that finding ways to prevent the occlusion of bypass grafts could contribute to development of treatments for vaospastic diseases not just for SVG disease.

Three pathophysiological changes found in SVG diseases —thrombosis, intimal hyperplasia, and atherosclerosis— are distinct but interrelated. The process of thrombogenesis is initiated by platelet activation. In physiological conditions, platelets activation is inhibited by nitric oxide and/or prostaglandin I_2 released from endothelial cells[7]; however, the endothelial cells of SVG are damaged

or lost during the operation.[5] There is a general agreement that the etiology of SVG diseases may be caused by the endothelial dysfunction and subsequent platelet aggregation.[7,8] Therefore, the use of substances that have anti-platelet activity may contribute to the reduction in the incidence of peri- and postoperative ischemic complications. Recently, accumulating evidence has suggested that anti-platelet therapy is beneficial for maintaining graft patency and blood flow.[9-11] The activated platelets release chemical mediators, such as adenosine diphosphate, thromboxane A_2, and 5-hydroxytryptamine (5-HT), that further activate peripheral platelets and constrict a blood vessel. Among these mediators, 5-HT has been identified to play a critical role also in the pathogenesis of vascular spasm.[5,12-15]

Ocean resources are common foodstuff in the Japanese diet and are considered beneficial for human health. The worldwide consumption of seafood has increased owing to the rising interest in its health benefits, especially in the economically advanced countries. Historically, many molecules that contribute to the preservation of health have been identified from ocean resources. In particular, fish oil, which contains a large amount of n-3 polyunsaturated fatty acids, is the most investigated bioactive ingredient.[16,17]

So, we have conceived this study in order to examine the further availability in health benefit of seafood. The aim of this study was to look for functional foods which contribute to prevent

5-HT-induced vasospastic diseases from ocean resources in Japanese coastal waters. To accomplish our aim, we evaluated the effects of ocean resource extracts on 5-HT-induced platelet aggregation and vasoconstriction in humans.

Materials and methods

Extraction of the water-soluble fraction from ocean resources

First, crude extractions of water-soluble fractions from five ocean resources were prepared for the screening test to find out effective seefood. A total of 100 g of freeze-dried samples of ocean resources, including orange roughy (*Hoplostethus japonicas*), 2 kinds of grenadier (*Caelorinchus kamoharai* and *Hymenocephalus lethonemus*), jack-knife prawn (*Haliporoides sibogae*), and round herring (*Etrumeus teres*), was homogenized in 1 L of ethanol. Each homogenate was centrifuged for 5 min at 1,600 × g, and sediment was suspended and re-homogenized in 1 L of distilled water. The resulting homogenate was centrifuged for 5 min at 1,600 × g. The supernatant was filtered and lyophilized to obtain the water-soluble fraction of each sample. The lyophilized extracts were weighed and dissolved in distilled water before use.

Plasmid construction

To visualize the 5-HT_{2A} receptor in viable cells, pTriEx4neo-5HT_{2A}-mECFP —an expression plasmid encoding the cyan fluorescent protein-fused 5-HT_{2A} receptors (5-HT_{2A}-CFP)— was constructed as previously described[18], except for the use of CFP instead of YFP.

Fluorescence measurements in HEK293 cells

HEK293 cells were maintained using the method by Nakai *et al.*[19] To express 5HT_{2A}-CFP, the cells were transfected with pTriEx4neo-5HT_{2A}-mECFP (1 μg/3.5-cm dish) using FuGENE 6 (Roche, Basel, Switzerland) according to the manufacturer's instructions. The transfected cells were incubated at 37°C for 1–4 days before testing. After replacing the culture media with a HEPES-buffered saline (HBS) solution containing 107 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 11.5 mM glucose, and 20 mM HEPES (pH 7.4), which was pre-warmed to room temperature, the cells were viewed under the microscope.

Cells expressing 5-HT_{2A}-CFP were monitored by excition at 458 nm and detection at 500 ± 25 nm with a confocal laser scanning microscope (LSM510Meta, Zeiss, Oberkochen, Germany). To observe changes in the localization of 5-HT_{2A}-CFP, 50 nM 5-HT was bath applied to the transfected cells. Images were taken at 5-s intervals.

To test 5-HT-induced transient changes in intracellular Ca^{2+} concentration (Ca^{2+} transient), the

HEK293 cells were transfected with pTriEx4neo-5HT$_{2A}$-mECFP and loaded with Fluo3-AM (Dojindo, Kumamoto, Japan), a Ca^{2+}-sensitive fluorescent dye, and 50 nM 5-HT was bath-applied. Images were taken at 5-s intervals, as previously described.[18,19]

Preparation of blood vessels and contractile studies

Human SVs were obtained from patients who had undergone CABG at the Miyazaki Prefectural Nobeoka Hospital (Miyazaki, Japan) or surgical varicose-vein treatment at the Kuwabara Clinic (Miyazaki, Japan). In the Miyazaki Prefectural Nobeoka Hospital, portions of each SV were sectioned into the desired lengths for bypassing the occluded coronary arteries, and the remainders were used for the experiments. In the Kuwabara Clinic, portions of SV with varicose-veins in the lower extremity were sectioned during surgical treatment. We used only tiny portions of each SV that were not extended. After each vein was obtained, it was immediately placed in a modified Krebs buffer, which had been previously aerated with 95% O_2-5% CO_2, and transported to our laboratory. The composition of the modified Krebs buffer solution was as follows (in mM): NaCl, 118.0; KCl, 4.7; $NaHCO_3$, 25.0; $MgSO_4$, 1.2; KH_2PO_4, 1.1; $CaCl_2$, 2.5; EDTA, 0.01; and glucose, 11.0. The final pH of the buffer was adjusted to 7.4. The ring segment of the SV was prepared as described previously.[20,21] The SV vessel was carefully sectioned into 2-mm rings, which were mechanically

denuded of endothelium. Each ring was suspended between stainless steel hooks in a 5-mL organ bath containing the modified Krebs buffer maintained at 37°C and continuously aerated with 95% O_2–5% CO_2. One hook was connected to a force transducer (Nihon-Kohden, Tokyo, Japan) to record the isometric tension in a computer system (PowerLab8/30, Bio Research Center Co., Ltd., Nagoya, Japan). The SV ring was stretched progressively to obtain the optimal resting tension (2.0 g) and allowed to equilibrate for 1 h. The buffer was changed every 30 min. Thereafter, the ring segment was assessed for the presence of any functional endothelium.

The SV ring was washed twice with the fresh modified Krebs buffer solution and preincubated for 30 min in the presence (ranging from 0.0003% to 0.003%) or absence of crude water-soluble jack-knife prawn extract (WJPE). Subsequently, the ring was stimulated by adding 30 μM 5-HT, and isometric contractive tension was monitored. The constriction of each ring was evaluated as a percentage of the constriction of WJPE-untreated control.

Purification of the WJPE

Purification of the crude sample was performed by HPLC with a reverse-phase column (6.0 i.d. × 250 mm; Capcell Pak UG120 C18, Shiseido Co., Tokyo, Japan), using a linear gradient elution system from H_2O to 80% acetonitrile for 40 min (injection amount, 1 mL of 30 mg/mL; flow rate, 4

mL/min; wave length, 215 nm). The fractions were assessed again using 5-HT_{2A}-CFP-expressing HEK 293 cells (described in the previous section), and fractions with activity, which were collected during retention times of 9–12 min, were evaporated, weighed, and stored at -80°C until use. The lyophilized extracts were re-dissolved in distilled water and subjected to the following tests.

Platelet aggregation test

Human platelets were obtained from fasted healthy volunteers. Freshly drawn venous blood was collected into 0.1 volume of 3.8% sodium citrate. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were obtained by centrifugation for 15 min at 170 × g and for 10 min at 1,600 × g, respectively. The platelet count in PRP was adjusted to 2.5×10^{11}/L with PPP.

Platelet aggregation was assayed with a PA-20 aggregation analyzer (Kowa, Tokyo, Japan), which determines the degree of aggregate formation based on the value of optical density. PRP (210 μL) was placed in a cuvette and incubated for 5 min at 37°C with 30 μL of aqueous solution of purified jack-knife prawn extract (final concentration, 0.1% or 0.3%) or distilled water for the control. Subsequently, 30 μL each of collagen solution and 5-HT (Sigma Chemical Co., St. Louis, MO; final concentration, 1.0 mg/mL and 1.0 μM, respectively) was added, and aggregate formation was monitored for 5 min. Platelet aggregation was evaluated using the optical density method.[12] The

suspension mixture was continuously stirred with a magnetic stirrer bar through the assay.

Ethics

Both of the ethics committees of the Miyazaki Prefectural Nobeoka Hospital and Kyushu University of Health and Welfare approved this study. Number of acceptance is 10-005 (study using human platelet) and 09-004 (study using human saphenous vein). All the patients and healthy volunteers submitted written consent to participate in the study. These experiments were performed in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for Experiments Involving Humans.

Statistical analysis

All the data are presented as mean ± standard error of the mean (SEM). In the fluorescence assay study, values are expressed as the mean ± SEM for 30–50 cells. Statistical comparisons were made using one-way analysis of variance (ANOVA) followed by the Dunnet post hoc test (examination of SV vasoconstriction) or the Newman-Keuls multiple range test (the other examination), with $P < 0.05$ considered statistically significant.

Statistical analyses were performed using the Statistical Package for Social Science (SPSS) 21.0J

for Windows.

Results

Assessment of the inhibitory effect of the water-soluble ocean resource crude extracts on 5-HT-induced calcium influx

First, we determined whether the 5-HT_{2A} expression in the HEK293 cells was functioning adequately. The CFP fluorescence, conjugated with 5-HT_{2A}, in the HEK293 cells was mainly observed in the plasma membrane (Fig. 1A, green). Then, 50 nM 5-HT-induced Ca^{2+} transients were observed in the CFP- positive cells (Fig. 1A, red). As shown in Fig. 1B, the Ca^{2+} concentration reached the maximum 5 s after application of 50 nM 5-HT and returned to baseline within 60 s.

To evaluate the effect of the water-soluble ocean resource crude extracts on the response to 5-HT, we investigated the effect of the crude extracts on the level of the 5-HT-induced Ca^{2+} influx in the HEK293 cells expressing 5-HT_{2A}-CFP. The crude WJPE significantly reduced the maximal Ca^{2+} influx. On the contrary, among the other extracts, only the *Hymenocephalus lethonemus* extracts significantly enhanced the maximal Ca^{2+} influx (Fig. 1C). The inhibitory effect of the crude WJPE on the Ca^{2+} influx was concentration-dependent (Fig. 1D).

Effects of crude WJPE on 5-HT-induced vasoconstriction

Next, we investigated the inhibitory effect of crude WJPE on endothelium-denuded human SV constriction induced by 30 μM 5-HT (Fig. 2). The maximal 5-HT-induced constriction of SV treated with 0.0003%, 0.001% or 0.003% was 91.6%, 66.6% or 55.1% of untreated control, respectively. Concentrations above 0.001% crude WJPE significantly inhibited 5-HT-induced SV constriction.

Effects of purified WJPE on 5-HT-induced platelet aggregation

Dissolved crude WJPE with distilled water is colored solution and is unsuitable for our platelet aggregation test. It is because we examine platelet aggregation by an optical-density method. So, we used purified WJPE for evaluation of its inhibitory effect on the platelet aggregation. The crude WJPE was fractioned by HPLC, and additional assessment of each fraction for the response to 5-HT was performed (data not shown). The fractions collected from retention times of 9–12 min, when the strongest reactivity was achieved, were subjected to the following studies as purified WJPE.

Then, we investigated the effect of purified WJPE on human platelet aggregation induced by 1.0 μM 5-HT. The purified WJPE pretreatment significantly reduced the platelet aggregation in a concentration-dependent manner (Fig. 3). Notably, pretreatment with 0.3% purified WJPE inhibited the platelet aggregation almost completely.

Discussion

In the present study, we found that WJPE reduced the response to 5-HT using the screening method of Ca^{2+} mobilization in 5-HT_{2A}-CFP expressing HEK293 cells. Furthermore, we confirmed the inhibitory effect of WJPE on 5-HT-induced constriction of SVs and platelet aggregation. These results suggest that jack-knife prawn contains active components to prevent vasospastic diseases including peri- and postoperative ischemic complications of CABG.

HEK293 cells don't have any types of 5-HT receptors, and the functions of specific subtype of 5-HT receptors can be evaluated by forcibly-expressing the receptors to HEK293 cells. So, we employed 5-HT_{2A}-CFP expressing HEK293 cells as a screening tool for determining the responsiveness to 5-HT via 5-HT_{2A} receptors. The 5-HT_{2A} receptor on the plasma membrane is coupled with the G_q/G_{11} protein, and the Ca^{2+} influx is evoked by 5-HT binding to the receptor. Stimulation with 0.1 μM 5-HT induced Ca^{2+} transients in the 5-HT_{2A}-CFP-expressing HEK293 cells (Fig. 1A). These results suggest that the transfected 5-HT_{2A}-CFP is functioning as well as wild-type 5-HT_{2A} receptor. Furthermore, the 5-HT_{2A}-CFP-expressing HEK293 cells were useful for evaluating the effects of the agents on the 5-HT_{2A} receptor-mediated responses. As shown in Fig. 1C, only the WJPE significantly reduced the 5-HT-induced Ca^{2+} mobilization in the 5-HT_{2A}-CFP-expressing

HEK293 cells, and the effects were observed to be concentration-dependent (Fig. 1D). Based on these results, we decided to investigate and evaluate the inhibitory effects of the WJPE on 5-HT-induced vasoconstriction and platelet aggregation in humans.

The WJPE inhibited the 5-HT-induced vasoconstriction (Fig. 2) in a similar way to 5-HT-induced Ca^{2+} entry into 5-HT_{2A}-CFP-expressing HEK293 cells (Fig. 1D). These results suggest that the screening method using 5-HT_{2A}-CFP-expressing HEK293 cells is an excellent way to estimate the effect of water-soluble materials on the 5-HT-induced responses. Platelet aggregation induced by 5-HT was also inhibited by purified WJPE, however, the concentration and pretreating time of WJPE was substantially different from vessel contractile studies. The reactivity of platelet samples decrease rapidly with time, so in this study, we pretreated platelet samples with WJPE for only 5 minutes. On the other hand, human SV samples were pretreated with WJPE for 30 minutes because of the reactivity of blood vessel is maintained for a long period of time. In our present study, the WJPE concentration that had a significant effect on 5-HT-induced vasoconstriction of SV and platelet aggregation is 0.001% of crude extract and 0.1% of purified extract, respectively (Fig. 2, 3). One of the reasons that higher concentration of WJPE is required to indicate the inhibitory effect on the platelet aggregation than the vascular constriction may be due to the short treatment time. The mechanisms of inhibitory effect of WJPE on the platelet aggregation and the vasoconstriction still

unclear, if it contained soluble molecules that bind to 5-HT, the WJPE would inhibit the action of 5-HT. Pretreatment with 0.001% purified WJPE, concentration that indicated the significant inhibitory effects on SV constriction (data not shown), did not affect platelet aggregation at all (data not shown). Assuming that the inhibitory effects of WJPE are caused by unidentified "trapping" molecules, this effective WJPE concentration in SVs should have some effects on platelet aggregation. Thus, the hypothesized 5-HT-binding molecules are not considered a cause of reduction in the response to 5-HT found in SVs and platelets.

We recently reported that treatment with insulin for 30 min reduced the 5-HT-stimulated vasoconstriction of human SVs. Moreover, we indicated that insulin induced internalization of the 5-HT_{2A} receptor.[19] Therefore, the reduction in the amount of 5-HT_{2A} receptor in the plasma membrane may have contributed to the decreasing responsiveness to 5-HT. However, the WJPE did not induce the internalization of the 5-HT_{2A} receptor expression in the plasma membrane (data not shown). Thus, the WJPE-induced poor responsiveness of human SVs and platelets to 5-HT was independent of the 5-HT_{2A} receptor internalization.

N-3 polyunsaturated fatty acids, such as the eicosapentaenoic and docosahexaenoic acids, rich in fish oil, demonstrate anti-platelet, anti-thrombogenic, anti-atherogenic, and vasohypotonic activities.[16,22-24] These fatty acids are also contained in crustaceans, including shrimps, as well as

marine fishes.[25] In our present study, we used aqueous jack-knife prawn extract. N-3 polyunsaturated fatty acids are lipophilic constituents and cannot be easily contained in aqueous extract. Therefore, the inhibitory effects of the WJPE on 5-HT-induced platelet aggregation and vasoconstriction demonstrated in this report were not induced by n-3 polyunsaturated fatty acids. Dietary shrimp consumption is effective for the prevention of ischemic heart diseases because it warrants the synergistic effects of n-3 polyunsaturated fatty acids and WJPE.[26]

In conclusion, we found that the aqueous constituents of the WJPE have inhibitory effects on 5-HT-induced vasoconstriction and platelet aggregation in humans. Jack-knife prawn is regularly-consumed shrimp for a long time in Japan. The mutagenicity and toxicity except in the case of anaphylaxis have never caused a problem. Therefore, jack-knife prawn could be one of a functional food with health-promoting benefits for most people with cardiovascular disorders including patients who have gone CABG. We hope that the results of this study lead to development of a remedy for cardiovascular diseases.

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Figure legends

Fig. 1

Effect of the water-soluble oceanic biomass extracts on the 5-HT-induced calcium influx. (A) Typical 5-HT-induced Ca^{2+} transient in the HEK293 cells expressing 5-HT_{2A}-CFP. Fluorescence images of cells preloaded with Fluo3-AM were taken before and after the application of 50 nM 5-HT. The numbers above the images indicate the length of 5-HT exposure in seconds. Green, fluorescence of 5-HT_{2A}-CFP; red, fluorescence of Fluo3. (B) Time course of the 5-HT-induced Ca^{2+} influx. Changes in the fluorescence of Fluo3 were measured before and after the application of 50 nM 5-HT. *ΔF/F*, change in fluorescence against its initial fluorescence. Bar, 5-HT application duration. (C) The inhibitory effect of the water-soluble oceanic biomass extract on the Ca^{2+} transient. The peak *ΔF/F* reflecting the peak change in the 5-HT-induced Ca^{2+} transient with pretreatment with water (control) or the extracts is shown. In this experiment, all the concentrations of the water soluble extract from ocean resources were 1.0%. The values are expressed mean ± SEM. $*P < 0.05$, versus the control. (D) The concentration-related effect of the crude WJPE on 50 nM 5-HT-induced induced Ca^{2+} mobilization.

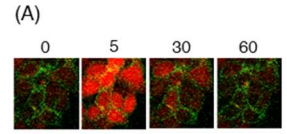
Fig. 2

The inhibitory effect of the crude WJPE on the 5-HT-induced constriction of denuded human SVs. Each vascular rings were preincubated for 30 min with or without crude WJPE. The constriction of each ring caused by 5-HT (30 μM) was evaluated as a percentage of the same concentration of 5-HT-induced constriction in control as 100%. The values are expressed mean ± SEM (n=3-5). $*P < 0.05$, versus the control.

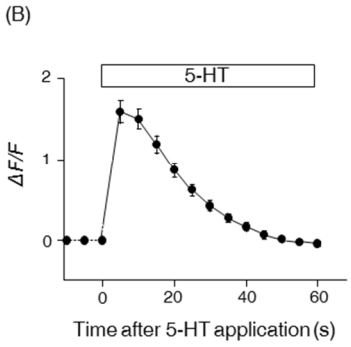
Fig. 3

Inhibitory effect of the purified WJPE on the 5-HT-induced aggregation of platelets harvested from humans. Platelets were obtained by mild centrifugation and preincubated for 2 min with or without purified WJPE. The degree of platelet aggregation caused by collagen (1.0 mg/mL) and 5-HT (1.0 μM) was evaluated as a percentage of the optical density of platelet-poor plasma (blank) as 100%. The values are expressed mean ± SEM (n=5).*$P < 0.05$, versus the control.

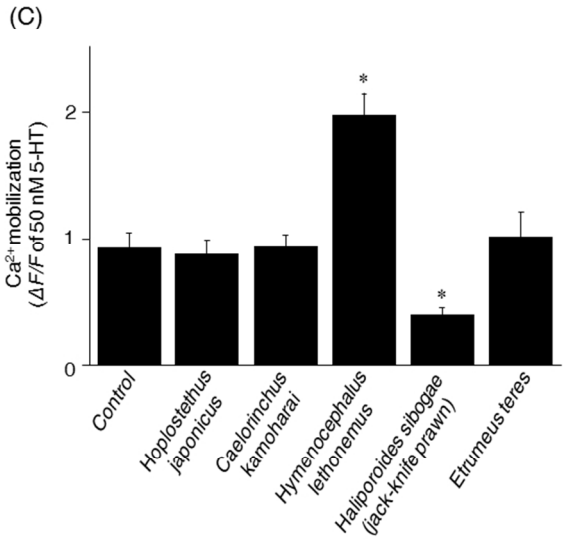
Fig. 1



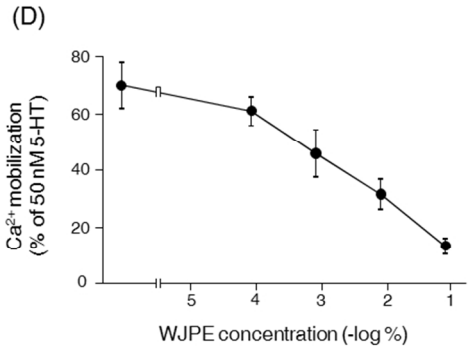
254x190mm (96 x 96 DPI)



254x190mm (96 x 96 DPI)

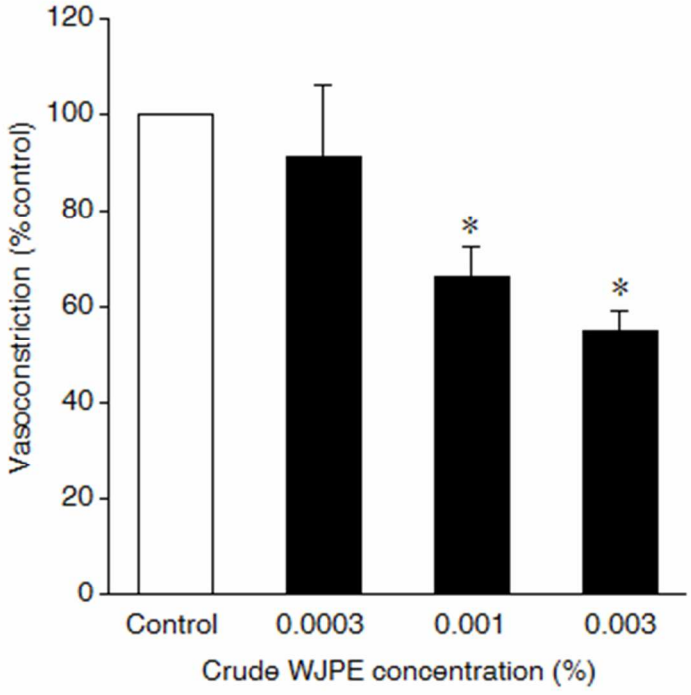


254x190mm (96 x 96 DPI)



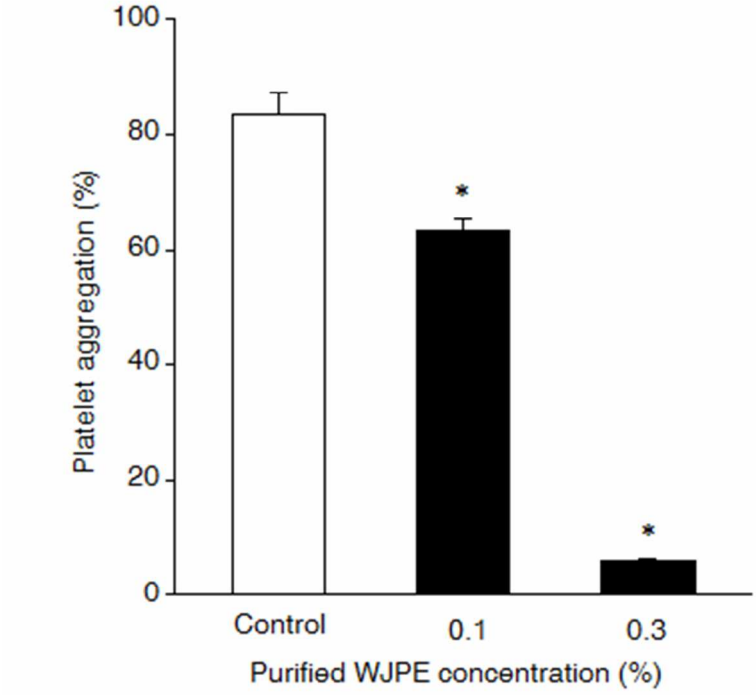
254x190mm (96 x 96 DPI)

Fig. 2



152x152mm (72 x 72 DPI)

Fig. 3



152x152mm (72 x 72 DPI)