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Vanillin as an Antidote for Box Jellyfish (*Chironex fleckeri*) Envenomation

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Abstract The phenolic compound, vanillin was tested and found to have potential anti-toxin activity against *Chironex fleckeri* toxin. Anti-toxin experiment conducted by premixing toxin and vanillin prior to intraperitoneal injection into mice showed that vanillin was able to completely neutralize the toxin in all 10 mice tested. It was also discovered that 0.5mg vanillin could 'rescue' 7 out of the 10 mice previously envenomed with 68μg/kg of toxin. Haemolysis assay performed showed vanillin was capable of neutralizing the haemolytic effect of the toxin in a concentration-dependent fashion. The IC_{50} value of vanillin incubated with toxin at dilution giving 50% lysis (EC_{50}) was determined to be 0.8mg/μg. Pharmacological studies using isolated rat aorta showed that 2μM of vanillin was able to completely reverse the contraction induced by 0.1mg/ml toxin.

Abstrak Kompaun fenolik, vanilin telah dikenalpasti berupaya bertindak sebagai anti-toksin terhadap bisa *Chironex fleckeri*. Eksperimen anti-toksin yang dilakukan dengan mencampurkan bisa serta vanilin sebelum disuntik pada perut tikus mencit menunjukkan vanilin berkebolehan meneutralisasikan sepenuhnya kesan bisa obor-obor ini dalam semua 10 tikus mencit yang diuji. Dos 0.5mg vanillin boleh menyelamatkan 7 daripada 10 tikus mencit yang terlebih dahulu telah disuntik dengan 68μg/kg bisa berkenaan. Di samping itu didapati bahawa vanilin berkemampuan meneutralisasikan kesan hemolisis bisa obor-obor dengan bersandarkan pola kepekatan. Nilai IC_{50} vanilin yang diinkubasikan bersama dengan bisa pada pencairan 50% kesan lisis (EC_{50}) ditentukan sebagai 0.8mg/μg. Penilaian farmakologi dengan menggunakan pencilan otot 'aorta' tikus kasturi menunjukan 2μM vanillin mampu bertindak sepenuhnya dalam mengawal kontraksi otot berkenaan yang diaruh oleh 0.1mg/ml bisa.

(Vanillin, *Chironex fleckeri*, jellyfish, envenomation, anti-toxin)

INTRODUCTION

Chironex fleckeri was named by Dr. Ron Southcott in 1956, after its discoverer, Dr. Hugo Flecker [15]. It is commonly known as the Northern Australian box jellyfish, *Chironex* box jellyfish, sea wasp, big stinger or sea stinger. This cnidarian belongs to class Cubozoa under the order Chirodropidae. *Chironex fleckeri* is quite abundant in Australian waters during the summer months especially at the west, north and northeastern region [29]. *Chironex* are mostly concentrated in shallow waters but have been found 100 - 300m out to sea, although rarely on the surface, and mainly in calm water [29]. Specimens resembling *Chironex fleckeri* have been identified from the following Indo-Pacific regions: Brunei, Malaysia, Philippines and Vietnam [29]. In Malaysia, death of a 10-year old schoolboy stung by a jellyfish identified as *Chironex fleckeri* had been reported by a local daily newspaper [1]. The incident happened in Tanjung Kubong beach, Labuan near Sabah. The authenticity on the identity of the reported jellyfish was unclear as *Chironex fleckeri* was not known to exist in these areas. It is likely that the jellyfish could have been from species more common in this area and resembling *Chironex*

fleckeri such as *Chiropsalmus quadrigatus* and *Chiropsalmus buitendijki.*

Chironex fleckeri has 4 pedalia, each with up to 15 highly retractile and extendable tentacles. Each tentacle can stretch up to 2 metres and contains many millions of nematocyst (or stinging cells) that discharge toxin through the skin on contact [14]. It is transparent in water and weighs up to 6kg and measures about 20 - 30cm across the bell [15].

Contact with its tentacles causes severe pain and extensive local swelling. Due to its abundance and the severity of its stings, it has become a major health problem to beach patrons in places where it is commonly found. There have been at least 63 confirmed deaths from envenomation by the cubozoan in the Indo-Pacific region [29]. In summer season of 1985 - 1986 alone, *Chironex* envenomations accounted for at least five deaths [20]. On an average, there have been one or two deaths each summer [30]. The onset of symptoms and death is usually very rapid. Some fatalities have been reported to occur less than 1 min after the time of first contact with tentacles [4, 28].

Ipomoea pes-caprae (L.) R. Br., a plant distributed on the seashores of both hemispheres throughout the tropics has been successfully used by Thai fisherman for the treatment of various types of inflammation including dermatitis caused by venomous jellyfish [22, 27]. An extract known as IPA obtained from petroleum ether extraction of a steam distillate of the leaves, exerted inhibitory effects on contractions of the isolated guinea pig ileum induced by different spasmogens, including jellyfish toxins [22, 27]. In addition, IPA prepared as 1% cream has also been tested in a preliminary clinical study in patients with various degrees of jellyfish-caused dermatitis [24]. The cream was effective for dermatitis, prevented serious wound formation, decreased tissue destruction and prevented recurrence of the symptoms. In another report, IPA was studied for its ability in neutralizing the toxic activities of various jellyfish toxins. The neutralizing ability assessed by observing the effect of IPA on jellyfish toxin-induced proteolysis and haemolysis. When IPA was incubated with active toxins, it inhibited the actions of all jellyfish toxin tested [23]. In our preliminary study, results showed the flower extract of this plant having had significant 'prophylactic' and 'rescue' capabilities in preventing the lethal action of *Chironex* toxin in mice. GCMS analysis of the active fraction from this extract showed the presence of vanillin. In the present study, the use of this phenolic compound as a possible antidote for Box Jellyfish envenomation had been demonstrated.

MATERIALS AND METHODS

Freeze-dried tentacles of *Chironex fleckeri* were kindly supplied by Dr. Phil Alderslade of the Museum of Arts and Sciences, Darwin, Northern Territory, Australia. Vanillin used in this experiment was of analytical grade and purchased from Merck (Germany). Sprague-Dawley rats and ICR mice were purchased from the Central Animal House, Faculty of Medicine, University of Malaya.

Toxin extraction from *Chironex fleckeri* tentacles

Crude jellyfish toxin extracts were prepared according to previously described techniques of [21] with a few modifications. Briefly, freeze-dried tentacles were re-suspended in cold deionised water (1:6) for an hour in ice. The tentacles were then homogenised till no large bits appear and sonicated three times for 15 - 20 sec cycles each interspersed by two minute intervals. This suspension was then clarified by centrifugation at 20,000 x g for ½ an hour at 4^0C. Multiple 1ml aliquots were stored at -70^0C until use.

Protein determination of *Chironex fleckeri* toxin

Quantitative estimation of protein in samples was done using the dye binding technique of Bradford [2]. One mg/ml of Bovine Serum Albumin (BSA) was prepared and used for the protein standard curve. In this study, macro Bradford assays was used where the concentration of BSA contains 10 to 100 µg of protein. 5ml of Bradford reagent was added to 100µl of crude toxin extract and standard solution. The mixture was then incubated at room temperature for 10 minutes. The absorbance of the protein was measured at 595nm. The protein content of the samples was determined from the standard curve.

Bioassay screening of anti-toxin activity in vanillin

For the bioassay procedures, 25 - 40g adult ICR mixed-bred un-anesthetized mice were employed in two separate experiments. For each experiment

a standard lethal dose (1100X dilution of the toxin extracted from *Chironex fleckeri* tentacles) was selected for routine use that would 'kill' a mouse within 2 - 3 minutes. In the first experiment, the toxin and the vanillin were mixed together prior to the introduction into the mice while in the other experiment the toxin was injected first before the introduction of vanllin. For the former, 200µl of vanillin-toxin mixture, obtained by adding together 100µl of toxin (550X dilution of the crude extraction of *Chironex fleckeri* tentacles) and 100µl of vanillin, was injected into the tail vein of mice. In the 'rescue' experiment, 50µl of toxin (1100X dilution of the crude extraction of *Chironex fleckeri* tentacles) was administered immediately (within two to three seconds) followed by intravenous injection of 100µl of vanillin over five to ten seconds. In both experiments, the vanillin was dissolved in 10% DMSO. The crude toxin was known to be labile to temperature and thus all experiments were conducted by storing the diluted toxin in ice throughout the duration of each experiment. At the same time the control mice were occasionally injected with the toxin preparation during the course of the experiment to ensure that the lethality of the toxin was still intact.

Toxicity study on vanillin

In this experiment, 3 sets of 25 - 40g ICR mice consisting of 5 animals per group were injected intravenously with different concentration of vanillin. Each group received 200µl of 1mg/ml, 5mg/ml and 10mg/ml concentrations of vanillin dissolved in 10% DMSO. Normal conditions were maintained whereby all the test mice were allowed food and water throughout the experiment.

Haemolytic assay

Overview

The assay was based on the haemolysis of erythrocytes following the method of Pongprayoon *et al.* [23] with slight modification. Haemolysis activity of the jellyfish toxin was obtained by determining the percentage of haemolysis of the erythrocytes compared to those incubated in 1mg/ml saponin solution (100% haemolysis) under the same conditions. The extent of haemolysis was determined spectrophotometrically by the amount of haemoglobin released into the supernatant.

Preparation of erythrocyte suspension

Rat erythrocytes were obtained by cardiac puncture of the animal under ether anaesthesia. Approximately 2.0 - 2.5 ml blood drawn was added into vacuum blood collection tubes that contained powder mixture of 4.0mg potassium oxalate and 5.0mg sodium fluoride as anticoagulant. The erythrocytes were then centrifuged immediately at 1000 x g for 5 minutes. The serum was discarded and the pellet of red blood cells was washed three times with Krebs-Henseleit solution of the following composition (g/l) KCl 0.35, NaCl 6.92, $CaCl_2.2H_20$ 0.37, $NaHCO_3$ 2.1, KH_2PO_4 0.16, $MgSO_4.7H_2O$ 0.29 and glucose 1.0. The washed erythrocytes that had been pelleted were taken as the 100% suspension. A 0.6% red blood cell suspension was prepared by diluting the 100% suspension in Krebs-Henseleit solution. Freshly prepared erythrocytes suspension was used for every experiment.

Assay procedure

The haemolysis assays were performed in duplicate by incubating 1.0ml of 0.6% erythrocytes suspension and 100µl of various concentration of toxin extract at room temperature for 30 minutes. Various concentrations of toxin were achieved by performing serial dilution of the crude toxin by using deionised water. Erythrocytes lysis of 100% was achieved by adding 100µl of 1mg/ml saponin solution to 1ml of 0.6% erythrocytes suspension. Centrifuging at 10,000 x g for 2 min terminated the reaction. The amount of haemoglobin released into the supernatant was estimated by measuring the absorbance of haemoglobin at 570nm.

Determining the neutralizing effect produced by vanillin

The neutralizing effect of vanillin was assessed by incubating 100µl of various concentrations of vanillin for 10 minutes with 100µl toxin at concentration giving 50% lysis (EC_{50}) in the absence of vanillin. After incubation, the mixture was assayed for haemolytic activity and the results were expressed as % inhibition with 100% denoting no remaining activity.

Pharmacological studies using smooth muscle

Male Sprague Dawley rat (200 - 250g) was killed by stunning. The abdomen was opened and the aorta was removed from the rat. The descending aorta was freed from connective tissue and cut

into 2 - 3 mm rings. The aorta ring was suspended in 2.5ml organ bath containing Krebs solution (g/l): NaCl (136.9 mM), KCl (5.4 mM), Glucose (5.5 mM), $NaHCO_3$ (23.8 mM), $CaCl_2$ (1.5mM), $MgCl_2$ (1.0 mM) and EDTA (0.001 - 0.01 mM). The baths were warmed to 37^0C and gassed with oxygen containing 5% CO_2. The solutions in the baths were changed every 20 - 30 minutes. All responses were recorded isometrically using a Maclab polygraph software. The aortic rings were equilibrated for 20 minutes before stretching to approximately 1g and allowed to further equilibrate for another 60 minutes before starting the pharmacological experiment. The smooth muscles were precontracted with Iso -72.7 mM K^+ solution (g/l): NaCl (69.6 mM), KCl (72.7 mM), glucose (5.5 mM), $NaHCO_3$ (23.8 mM), $CaCl_2$ (1.5 mM), $MgCl_2$ (1.0 mM) and EDTA (0.001 - 0.01 mM). The contraction by KCl is measured in gram tension and considered as 100% contraction. The organ bath was then washed with Krebs solution.

The antagonistic effect by vanillin towards *Chironex fleckeri* toxin-induced muscle contraction was tested as follows. The smooth muscle preparation was first treated with 100µl of toxin at concentration of 2.35 mg/ml, which is equivalent to 10X dilution of the crude toxin extraction. As the response curve slowly approached plateau, 200µl of 10% DMSO was added. Next, 200µl of vanillin in 10% DMSO at concentration of 5mg/ml was treated to the organ bath.

RESULTS

The protein concentration estimated in crude jellyfish extraction was 23.5 ± 0.3 µg/ml using the macroassay method of Bradford [2]. Table 1 showed the experimental results of anti-toxin activity produced by vanillin dissolved in 10% DMSO. The experiments using vanillin was performed by using 10% DMSO as this concentration of the solvent caused no discernible changes when injected into control mice. At the same time, the potency of the toxin was not affected since control mice injected with the mixture of toxin and 10% DMSO died with the normal toxic dose. The results showed vanillin having capabilities of completely neutralizing the toxin in mice injected with premixed toxin and the phenolic compound.

Anti-toxin activity was evaluated on commercially obtained vanillin. Vanillin was dissolved appropriately in 10% DMSO. The experiment consisted of *i.v.* injection of 200µl of vanillin-toxin mixture which was obtained by mixing together 100µl of a lethal dose of toxin and 100µl of 10mg/ml vanillin. Time of death of animals recorded together with their respective body weight. The data above represents the mean ± S. D. of these 2 parameters.

The results in Table 2 showed that 7 out of the 10 mice used in this experiment survived the toxic effect of the toxin. This experimental result demonstrated the efficacy of vanillin as a therapeutic agent for envenomed mice by producing 70% protection against the jellyfish envenomation. The effective dose of vanillin, which prevented death in a mouse that had already been challenged with 68µg protein/kg of toxin material was 0.5mg.

The 'rescue' experiment consisted of *i.v.* injection of 50µl of a lethal dose of toxin administered immediately (within two to three seconds) followed by *i.v.* injection of 100µl of vanillin (5mg/ml) over five to ten seconds. Time of death of animals recorded together with their respective body weight. The data above represent the mean ± S.D. of these 2 parameters.

As depicted in Table 3 a dose of up to 74mg/kg vanillin seemed to cause no discernible effect towards the animals throughout the 3 weeks the observation was carried out. Initially, all mice injected with vanillin showed involuntary movement i.e. trembling however normal behaviour resumed 10 to 15 seconds after the injection. Test on concentration higher than 10.0 mg/ml vanillin was unable to be carried out as vanillin was reaching saturation limit at this concentration in 10% DMSO.

Table 1. Neutralization of vanillin dissolved in 10% DMSO towards *Chironex fleckeri* toxin

TREATMENT	LETHALITY DEAD/TOTAL	WEIGHT (g) MEAN ± S.D.	DEATH TIME (sec) MEAN ± S.D.
10% DMSO	0/10	29.9± 1.8	-
Toxin and 10% DMSO	10/10	26.0± 6.5	122.6± 61.1
Toxin and vanillin in 10% DMSO	0/10	27.7± 2.5	-

Table 2. 'Rescue' experiment of vanillin dissolved in 10% DMSO towards *Chironex fleckeri* toxin

TREATMENT	DEAD/TOTAL (<$^1/_2$hr)	DELAYED DEATH (>$^1/_2$hr)	WEIGHT MEAN ± S.D.	DEATH TIME (sec) MEAN ± S.D.
Toxin only	10/10	0	27.0± 2.9	148.8± 31.8
Toxin followed by vanillin	3/10	2	30.0± 2.4	117.7± 30.7

Table 3. Effect of vanillin in mice

CONCENTRATION (mg/ml)	OBSERVATION (TOTAL MICE = 5)	WEIGHT (g) MEAN ± S.D.
1.0	-survive-	26.7 ± 2.3
5.0	-survive-	22.6 ± 3.2
10.0	-survive-	27.2 ± 2.3

A volume of 200µl of various vanillin concentrations was injected into the tail vein of 3 sets of mice and left under normal conditions with food and water throughout the observation. Vanillin was dissolved appropriately in 10% DMSO prior to injection. The body weights of the animals were recorded. The data above represent the mean ± S.D. of this parameter.

The *Chironex fleckeri* toxin induced haemolytic activity is as shown in Figure 1. Since the slope of the curve in the 50% haemolysis region is steeper than in the 100% haemolysis region, routine test adaptation of a visual end point of > 50% haemolysis was found more precise than using an end point of 100% haemolysis. *Chironex fleckeri* toxin tested showed high haemolytic activity with EC_{50} of 4.7µg/ml as determined from the graph.

Haemolytic activity of the jellyfish toxin was obtained by determining the percentage of haemolysis of the erythrocytes compared to those incubated in 1mg/ml saponin solution (100% haemolysis). The amount of haemoglobin released into the supernatant was estimated by measuring the absorbance of haemoglobin at 570nm. The data above represent the mean ± S.D. of 4 experiments.

When vanillin was incubated with the toxin, vanillin was found to neutralize the toxin's effects in a concentration-dependent fashion as shown in Figure 2. The IC_{50} value as determined from the graph was 0.8mg/µg. Inhibition of haemolysis by higher concentration of vanillin was not determined as the compound was unable to dissolve further in 10% DMSO.

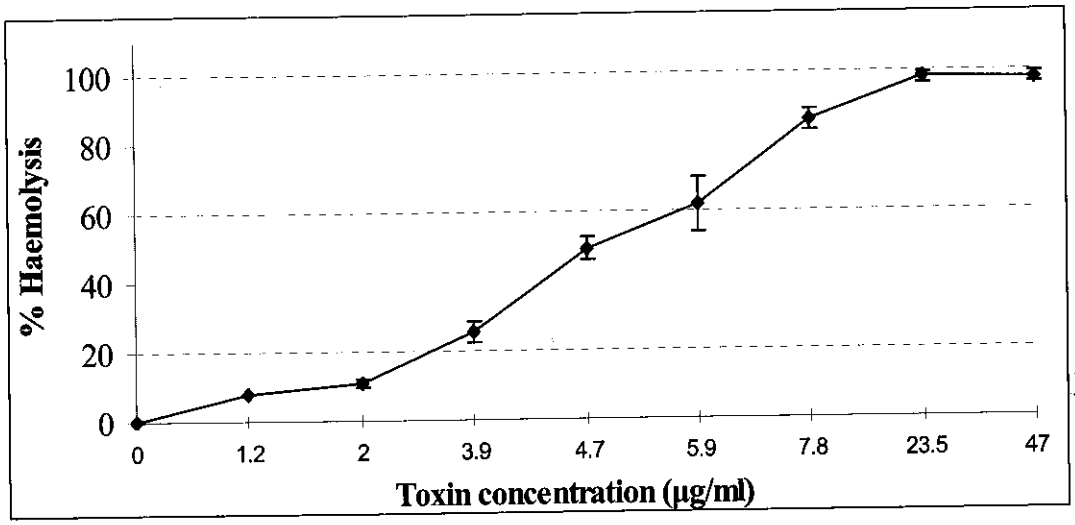

Figure 1. Haemolysis of rat red blood cells by various concentration of crude *Chironex fleckeri* toxin

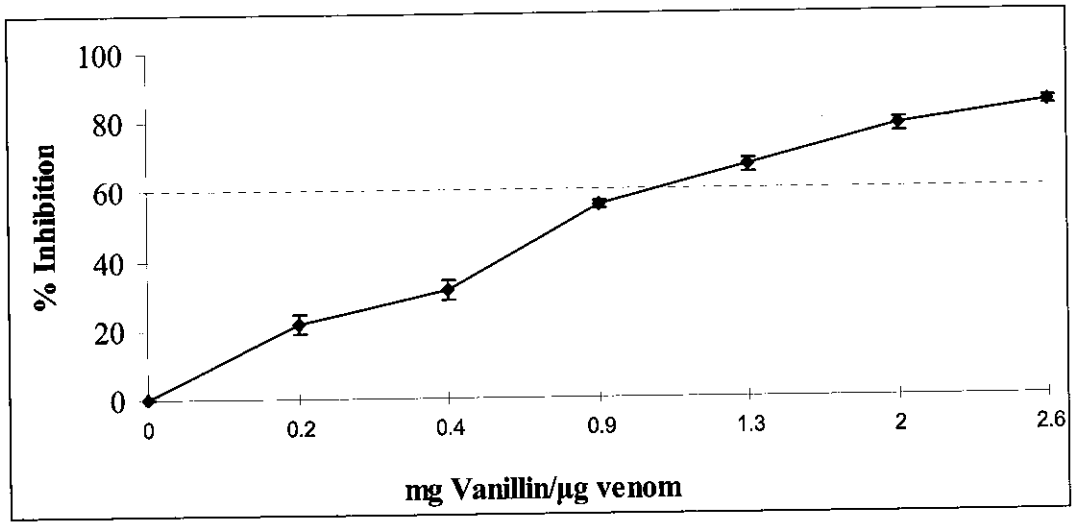

Figure 2. Neutralization of *Chironex fleckeri* toxin-induced haemolysis by various concentration of vanillin

The neutralizing effect of vanillin, assessed by incubating various concentrations of vanillin for 10 minutes with toxin at concentration giving 50% lysis (EC_{50}) in the absence of vanillin. After incubation, the mixture was assayed for haemolytic activity and the results were expressed as % inhibition, 100% inhibition denotes no remaining activity. The data above represent the mean ± S.D. of 3 experiments.

Antagonistic effect by vanillin towards toxin-induced muscle contraction was conducted on isolated rat aorta preparation. In comparison to contraction given by high K^+ (as shown in Figure 3) the jellyfish toxic material induced a slower contraction of the muscle, but the spasm remained much longer even after repeated washings. Moreover, an isolated rat aorta ring could stand many experimental tests with high K^+ than with jellyfish toxic material, in which the musculature ultimately became paralyzed in the relaxed state after a few experiments. The toxin induced a maximum response of muscle contraction in about 10 min. Addition of 10% DMSO at the point where the maximum response reached plateau showed no discernible changes towards the muscle contraction. Next the antagonistic effect of vanillin was evaluated and

it was found that 2μM of vanillin was able to decrease completely the percentage of maximal response induced by 0.1mg/ml *Chironex fleckeri* toxin. The maximal response by the toxin was determined to be 107% in comparison to high K^+(72.7mM) that was assigned as 100% contraction.

Figures 3(a), 3(b) and 3(c) represent tracings of 3 similar experiments that were repeated for this study. The isolated rat aorta for each experiment was precontracted with high K^+ followed by washing with Krebs solution. The contraction by high K^+ is measured in gram tension and considered as 100% contraction. The pharmacological effect produced by vanillin towards the lethal dose of *Chironex fleckeri* toxin was tested by first contracting the smooth muscle with 100μl of toxin at concentration of 2.35mg/ml followed by addition of 200μl of 10% DMSO and lastly the organ bath was treated with 200μl of vanillin in 10% DMSO at concentration of 5mg/ml.

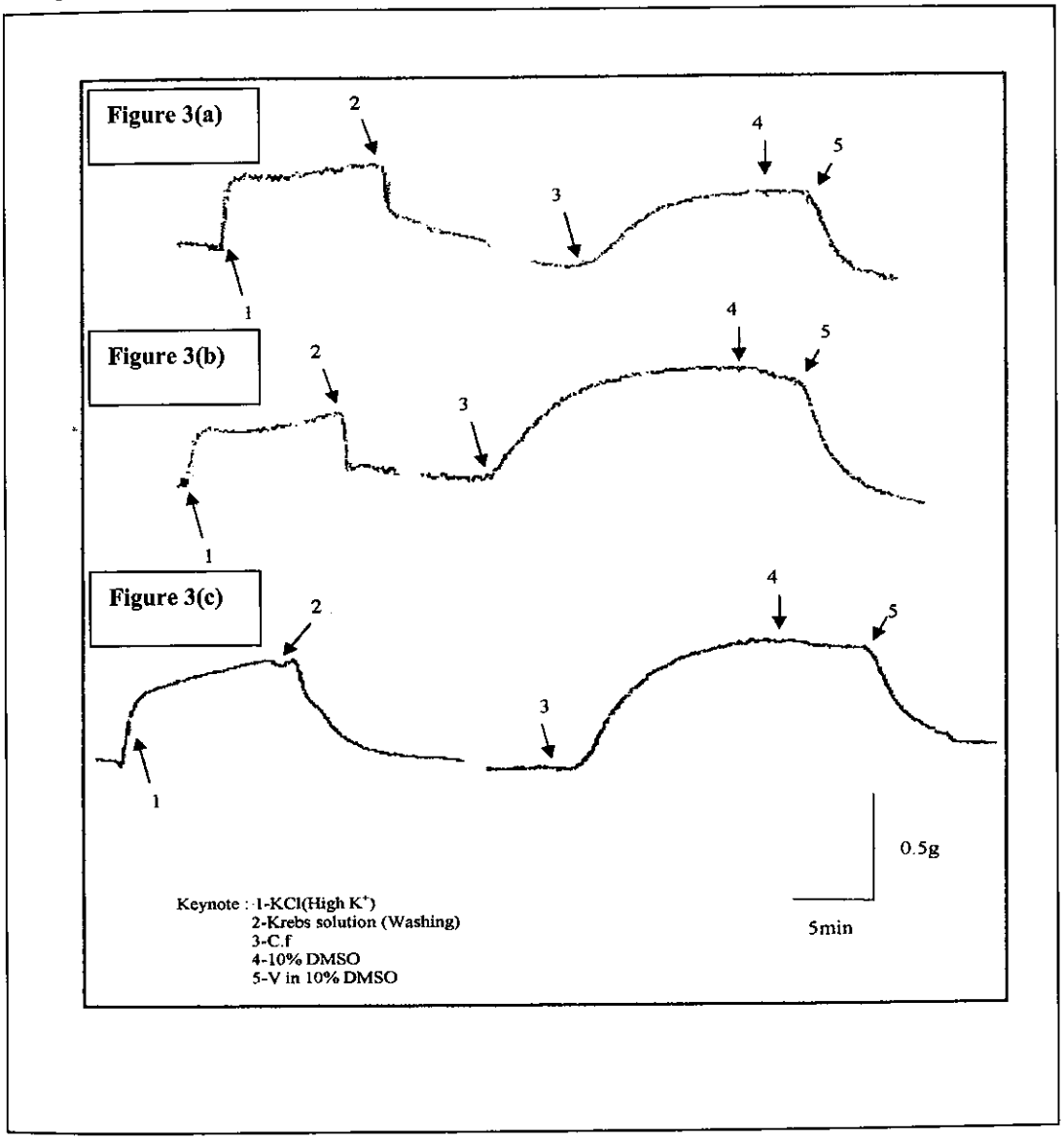

Figure 3. Tracings showing response of vanillin (V) towards *Chironex fleckeri* toxin (C.f) induced spasm in rat aorta

DISCUSSION

Bioassay antitoxin experiments on vanillin were conducted by injecting the toxin and sample into the tail veins of mice. This route of injection was used because it produces systemic effects whereas no systemic effects were produced when the nematocyst toxins were injected subcutaneously or intraperitoneally [12]. Serial dilution of the crude toxin with deionized water was carried out to decrease the amount of toxin in order to delay the time of death. The response in mice injected with the diluted toxin was similar to that effects observed previously [7]. Almost immediately after *i.v.* injection of toxic samples, violent convulsions were observed in the experimental mice. The legs often were hyper-extended. Paralysis and death usually occurred within 3 minutes after injection.

Antitoxin investigations performed on vanillin showed that it had neutralizing ability in mice envenomed with *Chironex fleckeri* toxin. In both antitoxin experiments conducted in mice i.e. premixed toxin-vanillin and toxin followed by vanillin, the phenolic compound was dissolved in 10% dimethylsulphoxide (DMSO). This vehicle was chosen based on the knowledge that vanillin being less soluble in water (one gram of vanillin dissolves in 100ml water, 16ml water at 80^0C) as compared to various other solvents such as alcohol, chloroform, pyridine or oil [3].

Numerous studies conducted [6, 16, 26] concluded vanillin has very low toxicity, resulting it to be on the GRAS (generally regarded as safe) list and also accepted for food use by the FDA (Food and Drug Administration). All previous toxicity studies conducted on vanillin seemed to investigate the toxic effects of the compound administered orally and none reported effects of intravenous infusion. A study involving injection of vanillin into the caudal vein of mice was carried out and it was found that a maximum dose of up to 74mg/kg did not produce any adverse effect. This amount was found to exceed far beyond the maximum dose (17mg/kg) of vanillin ever used at any one time of the antitoxin experiments conducted in this study.

Chironex fleckeri toxin was known to contain haemolytic, dermatonecrotic and lethal factors. The haemolytic activity had been well demonstrated in nematocyst toxins and whole tentacle extracts of the box jellyfish [5, 10, 17, 18, 19]. It was also reported that an oil extract (known as IPA), extracted from leaves of the *Ipomoea pes-caprae* (L.) R. Br. plant, capable of neutralizing the haemolytic activity of toxins from specimens of the jellyfish genera *Cassiopeia*, *Cyanea* and *Mastigias* captured in the Gulf of Siam, Thailand [23]. By adopting the method used by Pongprayoon *et al.* [23], the neutralizing activity of vanillin towards *Chironex fleckeri* toxin induced haemolysis of red blood cells was investigated in the present study. We concluded from our study vanillin was capable of neutralizing the haemolytic activity of the whole tentacle extracts of the Australian box jellyfish in a concentration-dependent fashion.

Myotoxic material, which elicits powerful sustained contractures of crustacean and mammalian musculature, has been found in the crude toxin obtained from isolated nematocyst of *Chironex fleckeri* [8, 9, 12]. The present study showed vanillin was capable of antagonizing the contraction induced by *Chironex fleckeri* toxin on smooth muscle of rat aorta.

It is evident from the present study that the lethal factor of *Chironex fleckeri* toxin can be neutralized in experimental animals by an appropriate dose of vanillin. Whether or not this may be analogous to the human situation will probably depend on clinical trial in future studies. Evidence also suggested that this phenolic compound had significant ability to neutralize the hemolytic component in *Chironex feckeri* toxin. However the role of the haemolysin in *C. fleckeri* envenomation in humans is not of clinical significance as it is not the major contributing factor towards the severity of the envenomation. Nevertheless, an antivenom agent such as vanillin that possesses dual mechanisms of action could exhibit synergistic affect, thus increasing its effectiveness.

The box jellyfish antivenom was produced in Commonwealth Serum Laboratories (CSL), which was purified from sheep immunoglobutin in 1970 [25]. Since its introduction this antivenom has been widely used in Australia in over 300 cases of *Chironex* envenomation, with no untoward effects [13]. The antivenom has been used predominantly for severe stings that were life-threatening [13, 25, 31]. Standard procedures for immediate management of *Chironex* envenomation required the

administration of antivenom either intramuscularly or intravenously [14]. Nevertheless, this treatment just like any others has received countless negative reviews. Laboratory experiments with rats suggested that the antivenom was of little value in the recovery of mice after a previous, lethal intravenous injection of *Chironex* toxin [11]. In addition, the antivenom has been also shown to alleviate severe pain of less serious *Chironex* envenomation [31]. In 1988, the antivenom was withdrawn from surf clubs briefly with reasons, which among others includes its increasing cost and problems of availability [13]. If vanillin can be proven to be clinically useful, then it should be given serious consideration as an adjunct in envenomed victims of *Chironex fleckeri* since it is easily available and cost less than the CSL produced antivenom. Furthermore, as vanillin has already been considered as a food additive on the GRAS list and has little or no adverse effect at high doses in animals tested, it therefore qualifies easily as the next candidate for further evaluation as an agent for the treatment of jellyfish envenomed victims.

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