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Full Length Research Paper

Cytotoxic and anticoagulant peptide from *Scolopendra subspinipes mutilans* venom

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Scolopendra subspinipes mutilans **venom is a complex mixture of peptides and proteins with many biochemical and pharmacological activities. In this study, a novel peptide was identified by ultra filtration and reverse-phase high performance liquid chromatography (RP-HPLC). The amino acid sequence was FTGGDESRIQEG determined by Edman degradation. The molecular mass was 1296.05 Da determined by electrospray ionisation mass spectrometry (ESI-MS). The novel peptide displayed specific inhibitory effects on the proliferation of human liver cancer (HepG2) and human gastric cancer cells (MGC). It also showed antibacterial activity against the tested bacteria *Clostridium perfringens, Staphylococcus epidermidis,* and *Escherichia coli*. Moreover, this peptide prolonged the whole blood clotting time *in vivo*. This peptide is the first peptide showing cytotoxic and anticoagulant activities identified from *S. subspinipes mutilans* venom.**

Key words: *Scolopendra subspinipes mutilans* venom, cytotoxicity, antibacterial activity, clotting time.

INTRODUCTION

Centipedes are terrestrial arthropods belonging to the class Chilopoda, being characterized by the presence of a head and an externally segmented body containing a pair of articulate legs in each segment (Malta et al., 2008). About 2,800 species are known in the world, and several species are medically important (Malta et al., 2008). Centipedes are predatory, elongated and dorso-ventrally flattened arthropods. Venoms are extracted directly from the venom gland connected to the first pair forceps of centipedes to kill or immobilize prey as a defense mechanism against predators (Rates et al., 2007). Centipede envenomations are capable of inflicting severe symptoms in humans, including myocardial ischemia and infarction, hemoglobinuria and hematuria, hemorrhage and rhabdomyolysis, itching, fever and chills, general rash, eosinophilic cellulitis, and anaphylaxis (Mohri et al., 1991; Knysak et al., 1998; Rodriguez-Acosta et al., 2000). The long list of symptoms and complications induced by centipede envenomations suggests that centipede venoms contain a variety of different components with diverse functions (Mohamed et al., 1983; Fang et al., 1999).

You et al. (2004) isolated a 25 kDa serine protease from the venom of *S. subspinipes mutilans*, which demonstrated fibrinolytic activity by converting human Glu-plasminogen to activated plasmin. Wu et al. (2006) found that centipede acidic protein (CAP) significantly suppress the development of atherosclerosis, improves the hemorrheological disturbances and histopathological changes in the atherogenic diet fed rat model. Wenhua et al. (2009) isolated an antibacterial peptide named scolopendrin I from the venom of *S. subspinipes mutilans*. González-Morales et al. (2009) isolated a phosholipase A_2 from the venom of *S. viridis Say*. Peng et

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al. (2010) reported the structural and functional characterization of two antimicrobial peptides (scolopin 1 and 2) identified from the venoms of *S. subspinipes mutilans*. Rates et al. (2007) identified more than 60 proteins/peptides in *S. viridicornis nigra* and *S. angulata* venoms by a proteomic approach. Liu et al. (2012) further purified and characterized 40 proteins/peptides from crude venom of *S. subspinipes dehaani*. The purified proteins/peptides showed different pharmacological properties, including platelet aggregating, anticoagulant, phospholipase A_2, trypsin inhibiting, voltage-gated potassium channel, voltage-gated sodium channel, and voltage-gated calcium channel activities. Yang et al. (2012) identified 26 neurotoxin-like peptides from the venom of *S. subspinipes mutilans*.

The dried whole body of *S. subspinipes mutilans* L. Koch has been used for cancer treatment in traditional Chinese medicine for hundreds of years. The water extracts of the organism were reported to possess antitumor and immunopotentiating activities (Cohen and Quistad, 1998; Xu et al., 2010; Zhou et al., 2011; Zhao et al., 2012). Zhao et al.(2012) found that polysaccharide-protein complex from *S. subspinipes mutilans* could inhibit tumor growth *in vivo* by improving antitumor immune responses at least partly via down-regulating arachidonic acid metabolic pathways in tumor-associated macrophages. We recently identified an anticoagulant peptide from the *S. subspinipes mutilans* body (Kong et al., 2013). Cohen and Quistad (1998) found that centipede venom showed higher cytotoxic activities compared with spider venom. However, to our knowledge, the cytotoxic components in centipede venom remain largely unexplored.

In this study, a novel peptide was discovered and the amino acid sequence was determined by Edman degradation. This peptide displayed specific inhibitory effects on the proliferation of human liver cancer (HepG2) and human gastric cancer cells (MGC). It also showed antibacterial activity against the tested bacteria. Moreover, this peptide prolonged the whole blood clotting time *in vivo*.

MATERIALS AND METHODS

Venom collection

Adult *S. subspinipes mutilans* L. Koch (both sexes, *n* = 1,000) were from Chuzhou centipede farm (Anhui province, China). Venom was collected manually by stimulating the venom glands in the first pair forceps of centipedes using the multi-purpose electrical instrument with the conditions that the frequency was 7.8 ms (that is, 128 Hz). The voltage was 10 to 20 V, and the pulse width was 2 to 4 ms. Each milking occurred 1 week after the previous milking. Venoms were stored at –20°C until further use.

Peptide purification

The collected venom (400 mg) was diluted in 20 ml phosphate buffer (PBS), pH = 7.3 and then applied to an ultra filtration tube of 10 kDa, centrifuged at 10,000 rpm for 5 min. The low molecular weight fraction after ultra filtration was further purified by preparative-scale reverse-phase HPLC (BioLogic Duoflow System) on a Lichrospher C_{18} column (25 × 0.46 cm). Elution was performed using a gradient (0% B for 20 min, 0 to 60% B for 30 min) of 0.05% trifluoroacetic acid (TFA) in 5% acetonitrile (A) and 0.05% TFA in 95% acetonitrile (B) at a flow rate of 1 ml/min. The absorbance of elute was monitored at 214 nm.

Determination of molecular mass and peptide sequence

The molecular mass of the purified peptide was determined using an electrospray ionisation mass spectrometry (ESI-MS). Complete peptide sequencing was undertaken by Edman degradation on an applied biosystems pulsed liquid-phase sequencer, model 491.

Peptides synthesis

All peptides used for the following bioactivity assays were synthesized by the Fmoc (N-[9-fluorenyl]-methoxycarbonyl) chemistry in solid-phase synthesis. Usually, peptides are synthesized from the carbonyl group side (C-terminus) to amino group side (N-terminus) of the amino acid chain. The solid supports were preloaded with Wang resin for c-terminal acid in this synthesis. Protected amino acids were coupled by in situ activation with diisopropylethylamine (DIEA) and N-hydroxybenzotriazole (HOBt). Then dimethylformamide (DMF) with 20% piperidine was performed in deprotection for 20 min. Cleavage of the peptide from the Wang resin was performed by reagent (95% TFA/2.5% triisopropylsilane (TIS)/2.5% water) for 1 h. The synthesized peptides were purified by preparative-scale reverse-phase HPLC on a Kromasil C_{18} column (250 × 10 mm). Elution was performed using a linear gradient (30 to 100% B for 20 min) of 0.05% TFA in 5% methanol (A) and 0.05% TFA in 95% methanol (B) at a flow rate of 2 ml/min. The absorbance of elute was monitored at 214 nm. The main peak was pooled, dried in vacuum, lyophilized and stored at -20°C. The purity was analyzed by HPLC and ESI-MS.

Cytotoxicity of purified peptide on cancer cells

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay was used to measure the cytotoxicity of purified peptide on cancer cells (Wei et al., 2009). Briefly, murine prostate cancer cells (RM-1), human liver cancer (HepG2), human gastric cancer cells (MGC) and human umbilical vein endothelial cells (HUVEC) were grown in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with fetal bovine serum (FBS, 10%) (Gibco, Grand Island, NY, USA), gentamicin (25 mg/ml, Sigma) and L-glutamine (200 mM, Sigma), cultured in a humidified atmosphere of 5% CO_2 in air. Cells (4000 cells/100 µl/well) were seeded into a 96-well plate in triplicate. Peptides with concentrations of 6 to 256 µg/ml were added into the wells after 6 h. Non-treated culture cells were used as a negative control. After incubation with peptides for 72 h, the medium in each well was replaced with 20 µl of MTT with final concentration of 5 mg/ml. 150 µl diethylsulfoxide (DMSO)/well was added to dissolve the formed violet formazan crystals within metabolically viable cells after 4 h. The plates were incubated at 37°C for 15 min and then read at 570 nm with a microplate reader. Percent of growth inhibition was calculated as:

(OD of the control − OD of the experiment samples) / OD of the control × 100

IC_{50} was calculated as the concentration (µg/ml) of peptides causing

a 50% inhibition of cell viability.

In vitro antibacterial and antifungal activity test

Antibacterial activities of the purified peptide was determined against bacteria *(Staphylococcus aureus*, *Clostridium perfringens*, *Staphylococcus epidermidis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) and fungi (*Monilia albican, Aspergillus niger*) by an inhibition zone assay with some modifications as described previously (Lu and Chen, 2010). Briefly, agarose plates were seeded with microorganisms (about 10^6 cells in 10 ml of 1% agarose medium). Wells (3 mm in diameter) were punched out and a 5 µl peptide sample dissolved in water was loaded. Negative controls were loaded with water. After incubation overnight at 37°C, the diameter of the inhibition zone was determined. Liquid growth inhibition assay was further used for determination of the minimal inhibitory concentration (MIC). After microorganisms were grown to log phase, approximately 2000 bacteria or fungi were incubated with a series of peptide concentrations (512, 256,128, 64, 32, 16, 8, 4, 2, 1 and 0.5 µg/ml) in a 96-well microtiter plate for 24 h. The lowest concentration inhibiting bacterial growth was taken as the MIC value. Ampicillin and streptomycin sulfate were used as positive controls.

Hemolysis activity

Hemolysis assays were performed using human red blood cells (RBCs) in liquid medium as previous reported (Wei et al., 2006a, b). The RBCs were prepared from freshly collected blood by centrifugation at 4,000 rpm for 10 min. The cells were washed three times with 0.01 M PBS solution (pH = 7.4) and suspended as 1% suspension in PBS to prepare human erythrocyte solution. 100 µl of serial dilutions of the peptide (10, 40, 160, and 640 µg/ml) were added to 100 µl of human erythrocyte solution and incubated at 37°C for 60 min. The human erythrocyte solution were then centrifuged at 4,000 rpm for 10 min and measured at 540 nm with an enzyme linked immunosorbent assay (ELISA) plate reader. A parallel incubation in the presence of 0.1% (v/v) Triton X-100 was carried out to determine the absorbance associated with 100% hemolysis.

Clotting time *in vivo*

Whole blood clotting time (CT) in mice was measured by capillary glass tube method with some modification (An et al., 2011). All the experimental protocols to use animals were approved by the Animal Care and Use Committee at China Pharmaceutical University. Mice (18 to 22 g body weight) were divided into four groups (both sexes, six per group). Two groups were intraperitoneally injected with 16 and 8 mg/kg body weight of the purified peptide for 4 consecutive days. Other groups received normal saline and 12 mg/kg body weight of clopidogrel, respectively. One hour after the last administration, blood samples were collected through the retro-orbital plexus with a glass capillary and kept on a slide to allow for clotting. Stirring the blood with a dry needle every 30 s until needle wire can provoke a fibrous protein. So far, that is clotting time.

Statistical analysis

Data are shown as mean ± SE for the number of experiments indicated, and analysis of variance (ANOVA) followed by Tukey's tests were used for statistical comparison of the data. In all analyses, $P < 0.05$ was taken as statistically significant.

RESULTS

Peptide purification

The collected *S. subspinipes mutilans* venom (400 mg) was fractionated into the low molecular weight fraction (mainly peptides and small compounds) and high molecular weight fraction (mainly protein) by an ultra filtration tube of 10 kDa. The low molecular weight fraction was further purified by RP-HPLC and fractionated into ten peaks as illustrated in Figure 1. Fraction A showed cytotoxic activity.

Determination of molecular mass and peptide sequence

The purified peptide was collected, dried in vacuum and subjected to automated Edman degradation. This peptide was composed of 12 residues with an amino acid sequence as Phe-Thr-Gly-Gly-Asp-Glu-Ser-Arg-Ile-Gln-Glu-Gly (FTGGDESRIQEG). The molecular mass of the purified peptide was determined by ESI-MS, giving an observed molecular mass of 1296.05 Da (Figure 2). It matched the theoretical molecular mass of 1295.33 Da deduced by amino acid sequence of the purified peptide.

Peptides synthesis

All peptides used for the following bioactivity assays were synthesized by the Fmoc (N-[9-fluorenyl]-methoxycarbonyl) chemistry in solid-phase synthesis. The synthesized peptides were purified by RP-HPLC. The main peak was pooled (Figure 3). The purity was higher than 95% analyzed by HPLC. It is homologous to the purified peptide confirmed by ESI-MS.

Cytotoxicity of purified peptide on cancer cells

After incubation with 16 to 256 µg/ml peptides for 72 h, the proliferation of HepG2 and MGC cells were inhibited in a dose-dependent manner (Figure 4). The calculated IC_{50} value of peptide against HepG2 and MGC cells were 80 and 65 µg/ml, respectively. The survival ratio of cancer cells was decreased to about 10 to 40% after incubation with 256 µg/ml peptide for 72 h. However, RM-1 and HUVEC cells were not obviously affected (Figure 4). These results indicate that this peptide shows specific activities against cancer cells.

In vitro antibacterial and antifungal activity test

The MICs of purified peptide against bacteria and fungi were determined and summarized in Table 1. Among the

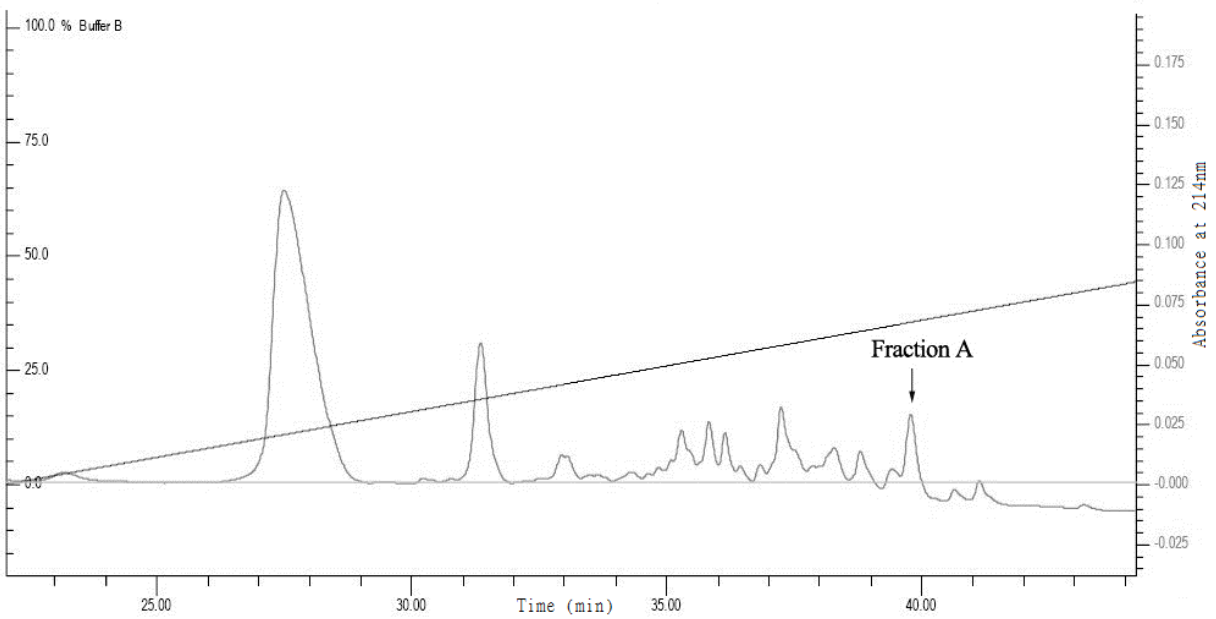

Figure 1. Purification of the cytotoxic and anticoaglant peptide from centipede venoms. The crude venoms were fractionated on a Ultra filtration tube. The low molecular weight fraction after ultra filtration was further purified by preparative-scale reverse-phase HPLC on a Lichrospher C_{18} column. The low molecular weight fraction of crude centipede venom was fractionated into ten peaks by RP-HPLC. Fraction A showed cytotoxic activity.

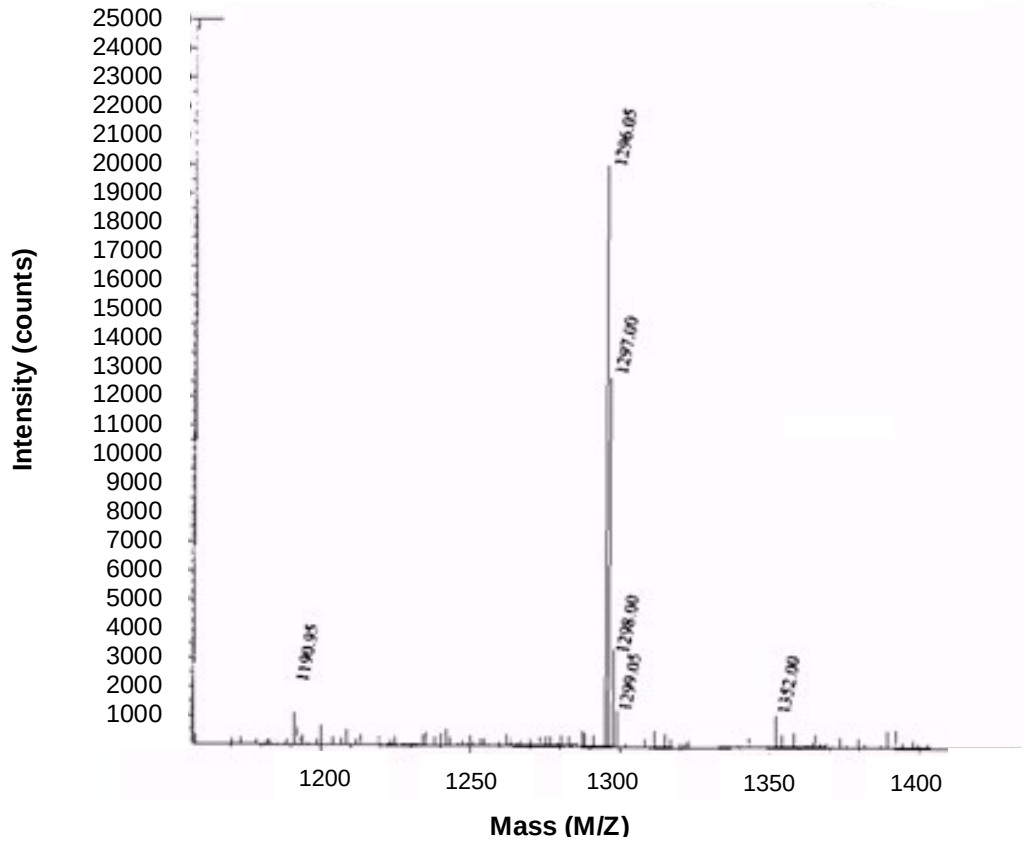

Figure 2. The molecular mass of the purified peptide determined by ESI-MS. The molecular mass of purified peptide is 1 296.05Da determined by ESI-MS. The 1297.0 m/z is peptide' isotope peaks

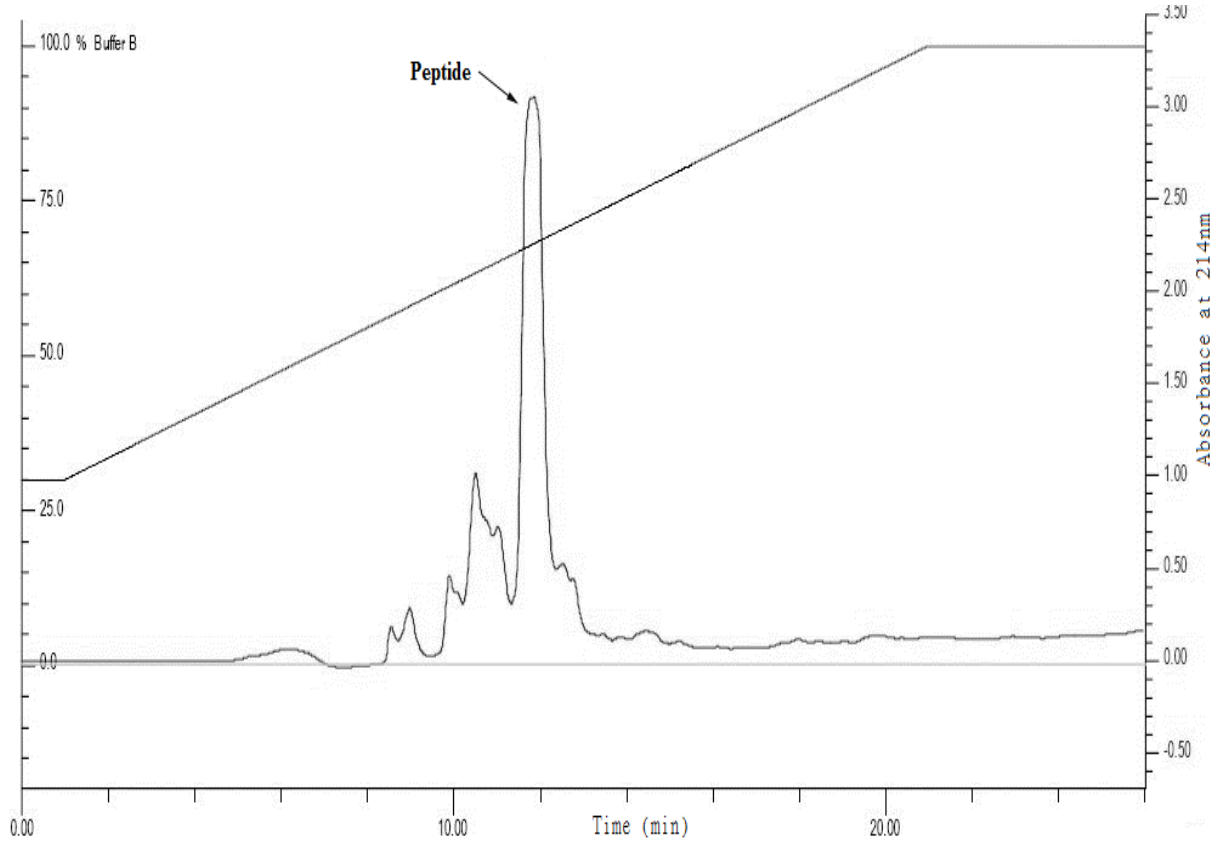

Figure 3. Purification of the synthetic peptide. The synthetic peptide solution was further purified by RP-HPLC on a Lichrospher C_{18} column.

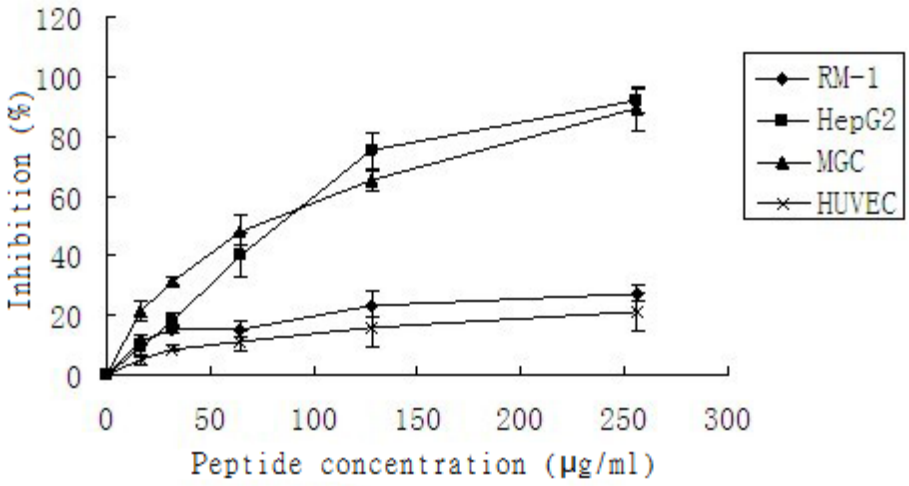

Figure 4. Cell inhibition curve as measured by MTT assays for purified peptide against cancer cell lines RM-1, HepG2, MGC and HUVEC cells, respectively. Error bars represent the standard deviation from the mean cell inhibition as determined by three independent experiments.

tested microorganisms, the purified peptide showed weak antimicrobial activities against *C. perfringens* and *E. coli,*

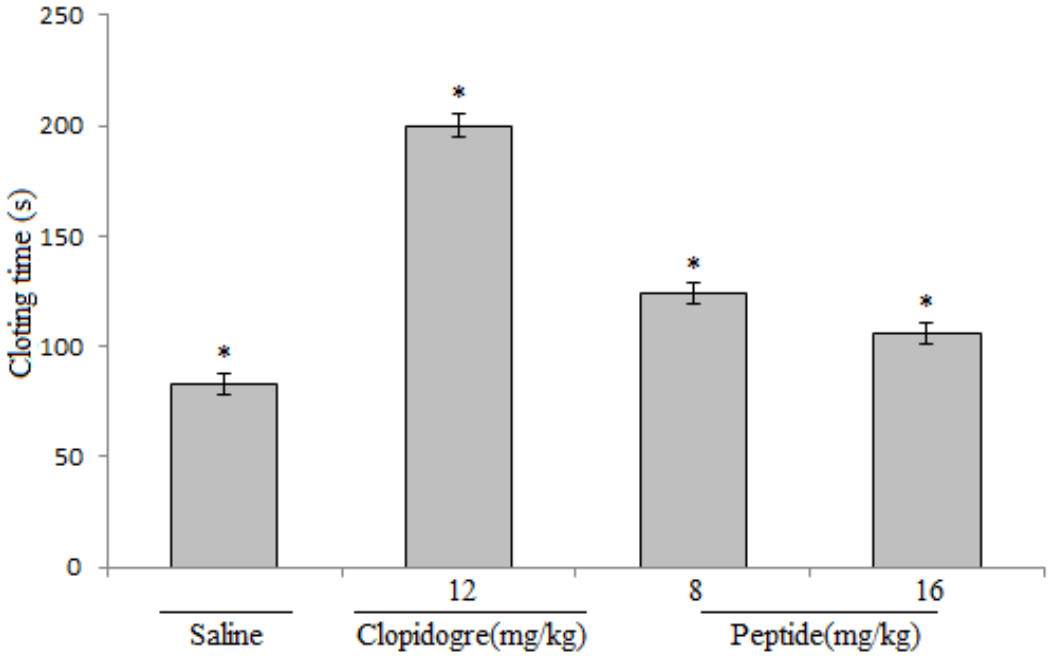

Figure 5. Clotting time prolonging activity of purified peptide. The peptide prolonged clotting time. Clopidogre as positive control. *P < 0.05 in comparison with those of control group (saline group).

Table 1. Antibacterial activities of purified peptide.

MIC (μg/ml)	*S. aureus*	*C. perfringens*	*S. epidermidis*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*
Peptide	NA	256	256	256	NA	NA
Ampicillin	0.5	0.5	2	16	>64	>64
Streptomycin Sulfate	8	16	0.5	>64	4	8

Ampicillin and Streptomycin sulfate are positive control.

and no activities against other microorganisms.

Hemolysis activity

When the purified peptide in gradient concentrations from 10 to 640 μg/ml was incubated with human RBCs, only about 0.01% hemolytic activity was observed after 1 h incubation at 640 μg/ml. Our results indicated the purified peptide was not toxic to human RBCs.

Clotting time *in vivo*

In comparison with the control group, 16 and 8 mg/kg group had significantly prolonged whole blood clotting time (P < 0.05) (Figure 5). It indicated that the purified peptide had anticoagulant effects.

DISCUSSION

Natural plants and animals are rich source for drug development (Atef et al., 2013; Tao et al., 2013; Malik et al., 2013). The dried whole body of *S. subspinipes mutilans* L. Koch has been used for cancer treatment in traditional Chinese medicine for hundreds of years. In this study, a novel peptide was identified by ultra-filtration and reverse-phase high performance liquid chromatography (RP-HPLC). The amino acid sequence was FTGGDESRIQEG determined by Edman degradation. The molecular mass was 1296.05 Da determined by electrospray ionisation mass spectrometry (ESI-MS). The novel peptide displayed specific inhibitory effects on the proliferation of human liver cancer (HepG2) and human gastric cancer cells (MGC). It also showed antibacterial activity against the tested bacteria *C. perfringens*, *S. epidermidis*, and *E. coli*. Moreover, this peptide prolonged

the whole blood clotting time *in vivo*.

Centipedes' venom are rich resource of peptides. Yang et al. (2012) identified twenty-six neurotoxin-like peptides from the venoms of *S. subspinipes mutilans*. Liu et al. (2012) also identified many peptides from another centipede, *S. subspinipes dehaani* venom. These peptides were found to act on voltage-gated sodium, potassium, and calcium channels, respectively. In the present study, we firstly identified a novel cytotoxic and anticoagulant peptide from *S. subspinipes mutilans* venom. The recent study of the closely related species *Scolopendra subspinipes dehaani* performed by Liu et al. (2012) uncovered multiple transcripts that contain the sequence of the described peptide, e.g: KC145039.1 .Given that centipede venoms are known to contain numerous proteases, the purified peptide may be a proteolytic fragment of centipede venom. Centipedes have been used for cancer treatment in traditional Chinese medicine for hundreds of years. Many components were identified from the centipede's body to be antitumor activity in several studies (Cohen and Quistad, 1998; Xu et al., 2010; Zhou et al., 2011; Zhao et al., 2012). We recently identified an anticoagulant peptide from the *S. subspinipes mutilans* body (Kong et al., 2013).

In the present study, we further confirmed that the centipede's venom also contains antitumor and anticoagulant components. However, the cytotoxic activity of the purified peptide is limited. The traditional usefulness of centipedes venom as anticancer agents may be due to indirect cytotoxity of this venom.

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