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SCREENING OF NOVEL CONOPEPTIDE FOR MOSQUITOCIDAL ACTIVITY AGAINST LETHAL MOSQUITOES (*Anopheles stephensi, Aedes aegypti*)

Nguyen Bao[1,2,*], Phan Thi Khanh Vinh[2], Nguyen Van Suu[1], Jean-Pière LE CAER[3]

[1]Institute of Biotechnology and Environment, Nha Trang University, 02 Nguyen Dinh Chieu, Nha Trang, Khanh Hoa province, Vietnam
[2]Faculty of Food Technology, Nha Trang University, 02 Nguyen Dinh Chieu, Nha Trang, Khanh Hoa province, Vietnam
[3]Institut de Chimie des Substances Naturelles, Centre de Recherche de Gif - FRC3115, UPR 2301, F-91198 Gif-sur-Yvette, France

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ABSTRACT

Mosquitoes are carriers of various diseases that result in millions of human deaths annually. However, the use of chemical mosquitocides has led to significant concerns, such as adverse effects on the environment, human health, and insecticide resistance. As a result, there is a pressing need for eco-friendly alternatives. This study aimed to identify potential mosquitocidal conopeptides from cone snail venoms targeting two lethal mosquito species, *Anopheles stephensi* and *Aedes aegypti*. Eleven *Conus* species were collected from Nha Trang Bay and their crude venoms were dissected and collected. Through cell survival and toxicity assays on mosquito larvae, five crude venoms were identified to have inhibitory effects. Further investigations involved fractionating these five crude venoms and testing them for cell cytotoxic activity were conducted. Four fractions from *Conus bandanus* venom, named CB37, CB39, CB41, and CB43, showed significant cytotoxic activity against cell survival. Other fractions from different *Conus* venoms also exhibited some cytotoxic activity at high concentrations but were not prioritised for further investigation. Toxicity experiments on adult mosquitoes revealed that the CB41 fraction had the most potential, with an IC_{50} of 30 μM. Additionally, this CB41 compound is a peptide in nature and was found to have a native mass of 3332.1 Da and three disulphide bridges. These results could deduce this long-chain peptide possessing a compact structure. To our knowledge, the conopeptide CB41 is reported first time to have mosquitocidal activity.

Keywords: *Conus*, mosquitocidal activity, venom, cone snails.

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[*]*Corresponding author email*: bao@ntu.edu.vn

INTRODUCTION

Mosquitoes cause millions of deaths every year, such as *Anopheles stephens*i (malaria), and *Aedes aegypti* (Dengue fever, Zika, Chikungunya, Rift Valley fever, Yellow fever) (WHO, 2016). There were 367,729 dengue cases and 140 deaths cumulatively registered in Vietnam (WHO, 2023). Both species are invasive in Vietnam and the control of these mosquitoes is essential. Chemical insecticides remain the dominant method for controlling mosquitoes in tropical and developing countries, like Vietnam. However, major problems with the use of chemical mosquitocides have arisen, such as environmental impacts, human health, and insecticide resistance (Kaur et al., 2024; Kumar et al., 2009; Van Wijngaarden Brock & Van Den Brink, 2005). A demand for eco-friendly mosquitocidal molecules is a serious requirement. In recent years, there has been growing interest of scientists in the potential of insecticidal peptides derived from the venom of animal predators such as spiders (Windley et al., 2012) and scorpions (Schwartz et al., 2012). These biological insecticides could allow for minimising ecological- and human health risks (Leng et al., 2011).

The peptides from *Conus* snails have been of particular interest to pharmacological properties as analgesic activities and their potential use in the treatment of human neurological diseases (Lewis et al., 2012; Olivera & Teichert, 2007). For instance, the omega-conotoxin MVIIA (Ziconotide) from *Conus magus* is applied as an analgesic drug (Wermeling, 2005). On the contrary, the potential applications of insecticidal peptides from *Conus* snail venoms are not yet well concerned, although their great variety and specificity towards invertebrate targets can make them appropriate candidates for insecticides, particularly mosquitoes. In addition, we have found some precious cone snails on the South-Central Coast of Vietnam. This study investigated target-oriented *Conus* peptides in the discovery of eco-friendly mosquitocides. This study aimed at screening cone-snail venoms to discover mosquitocidal peptide(s) against lethal mosquitoes (i.e., *An. stephensi*, *Ae. aegypti*).

MATERIALS AND METHODS

Conus snail collection

We harvested *Conus* species collected in the seawater of the Nha Trang Bay on the South-Central Coast of Vietnam. The *Conus* snail samples were carefully transported to the biological laboratory at Nha Trang University. They were carefully classified and identified according to their shape, patterns, and color (Nguyen, 2005). They were frozen at -80 °C. The crude venom of the whole venom apparatus of each species was carefully dissected, extracted with 0.1% TFA four times, lyophilized and then stored.

Fractionation of insecticidal crude venoms by RP-HPLC

The crude venoms were fractionated on HPLC system (Class-LC10, Shimadzu) with a C_{18} column (300 Å, 5 µm, L × I.D.25 cm ×10 mm) at flow rate o 1 $mL.min^{-1}$, with gradient program following as 0% B buffer in 10 min and 0-100% B buffer in 90 min. The peptides were detected at a wavelength of 220 nm. The peptide fractions of each venom were collected in tubes 50 mL to lyophilize. A buffer comprised 1,000 mL of distilled H_2O and 1 mL of trifluoroacetic acid (TFA), B buffer comprised 900 mL of acetonitrile, 100 mL of H_2O and 1 mL of TFA.

Rearing *Anopheles stephensi* and *Aedes aegypti*

Two wild-type mosquitoes (*An. stephensi*, and *Ae. aegypti*) were obtained from the Pasteur Institute in Nha Trang (Khanh Hoa, Vietnam). They were raised in a closed chamber at ~27 °C and 80% humidity under a 12-h light/12-h dark cycle and standard laboratory conditions. Mosquito larvae were fed with 0.04 mg ground fish food a larva/day. The adult mosquitoes were kept on 8% sucrose and were sometimes blood-fed with human blood and serum for egg production.

Screening of mosquitocidal fractions of *Conus* venoms

MTT assay to measure cell viability. The different cell lines were derived from the ovarian cells of *An. stephensi*, and *Ae. aegypti* (Pasteur Institute in Nha Trang, Vietnam). These cells were seeded in 96-well plates in 100 µL of culture medium (at 5,000-10,000 cells per well) with or without the venom or venom fraction. The microplate was incubated at 27 ^{o}C for 72 h. Ten microliters of the 12 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were added to each well and incubated at 37 ^{o}C for 4 h. After the removal of the MTT solution and the addition of 50 µL of dimethyl sulfoxide (DMSO), the plates were incubated for 10 min. The absorbance of DMSO extracts was measured at the wavelength of 540 nm using an enzyme immunoassay analyser. The final concentration of 50 µg.mL^{-1} blasticidin was used as the positive control. To calculate the inhibition activity of a venom or fraction on cell proliferation using the following formula:

$$\% \text{ inhibition} = [A_{540}(\text{DMSO}) - A_{540}(\text{sample})] * 100/[A_{540}(\text{DMSO}) - A_{540}(\text{blasticidin})]$$

The data was collected in Excel Microsoft 365 software. One-way ANOVA was performed by Microsoft Excel Analysis Tools.

For the mosquito larvicidal assay (WHO, 2005), the cytotoxicity venoms were dissolved in ethanol. A 5 µL aliquot of this solution was added to 5 mL of tap water containing 10 of 3–4th instar larvae of each treatment group. Tap water with the corresponding amount of ethanol/methanol was used as the negative/positive control groups, respectively. Larvae were observed periodically for behavioural effects at room temperature. The number of dead larvae was determined after 2, 4, 8, and 24 hours to monitor the larval mortality. Bioassays were carried out in triplicates using 100 larvae for each assay.

For the mosquito adult feeding assay (Francis et al., 2016), 5–7 day-old female mosquitoes (non-blood fed) were starved for 6 h and placed on ice for anesthesia, and then transferred to glass test tubes (n = 100). The solution of 10% sugar water with 0.5% ethanol was used as a negative control solution and dissolved fractionated purified conopeptides with serial dilutions to generate an appropriate number of concentrations. Cotton buttons were wetted with 1 mL of prepared solutions and inserted at the top of the test tubes. They were changed, and the solution was reapplied after 24 h. The test tubes were maintained at room temperature. Mosquitoes were observed periodically for behavioural effects. Mortality data were recorded after 24, 48, and 72 h with each experiment in three replicates.

The LC_{50} values, the concentration values for killing 50% larvae/adults at the end of experimentation, were calculated using Origin 6.0 software (OriginLab, Northampton, Massachusetts, USA). Data were analyzed using the Student t-test, and differences between the control and experimental values were considered statistically significant at $p < 0.05$.

Separation and purification of the mosquitocidal fraction

The bioactive fraction(s) were separated and further purified on an HPLC system with an analytical C_{18} column with a modified gradient program corresponding to each specific molecule. The samples were eluted at a flow rate of 1 mL.min^{-1} and detected at a wavelength of 220 nm. The fractions were collected minute by minute separately and accumulated in tubes 15 mL for validation of bioactivity, de novo peptide sequencing, and electrophysiological techniques (Yu et al., 2012). Each fraction was coded following the scientific name abbreviation of each species. For instance, CB10 means the fraction of LC eluant of *Conus bandanus* (CB), which was collected from the 10th minute to the 11th minute.

Reduction and alkylation for peptide sequencing

For complete reduction, an aliquot of the purified peptide (~0.13 nmol) was vacuum, dried, and reduced by 25 mM tris-2-carboxyethyl-phosphine in 0.5 M N-(2-

hydroxyethyl) piperidine-N"-2-ethanesulfonic acid buffer, for 20 min at room temperature, followed by alkylation with 50 mM iodoacetamide, for 30 min in darkness. Finally, the mixture was desalted by the ZipTip C_{18} column.

Mass spectrometry analysis

The purified sample (native- and completely reduced-alkylated forms) was analysed with a 5800 MALDI TOF/TOF™ Analyzer mass spectrometer (AB Sciex). The instrument is equipped with an Nd:YAG laser operating at 355 nm wavelength. Aliquots of each purified sample were mixed with 1 mL of a solution of 4 $mg.mL^{-1}$ of cyano-4-hydroxycinnamic acid. Acquisitions were performed in a positive reflection mode. Instrument calibrations were done using a peptide standard mixture. For tandem mass spectrometry (MS/MS) experiments, precursor ions were accelerated at 8 keV and the MS/MS spectra were acquired using 2 keV collision energy, with collision-induced dissociation (CID) gas at a pressure of 3.6 × 10-6 Torr. MS and MS/MS data of different-form peptides were processed using Data Explorer 4.9 (AB Sciex, France).

RESULTS

Screening of anti-mosquito *Conus* venom activity

Among these 11 snail species, there are rare snail species whose venom is rarely studied in the world, such as *Conus miles*, *Conus bandanus*, *Conus lynceus*, *Conus pulicarius* (Fig. 1). Figure 2 shows the toxicity of the crude venoms of the different *Conus* snails against *An. stephensi* cells and *Ae. aegypti* cells. The results demonstrated that five crude venoms from eleven species of snails had different inhibition phenomena for both types of cells. There was a 50–100% inhibition rate at 1 $mg.mL^{-1}$ venom concentration. These *Conus* extracts were of *Conus betulinus*, *C. lynceus*, *Conus eburneus*, *C. bandanus*, *Conus generalis*.

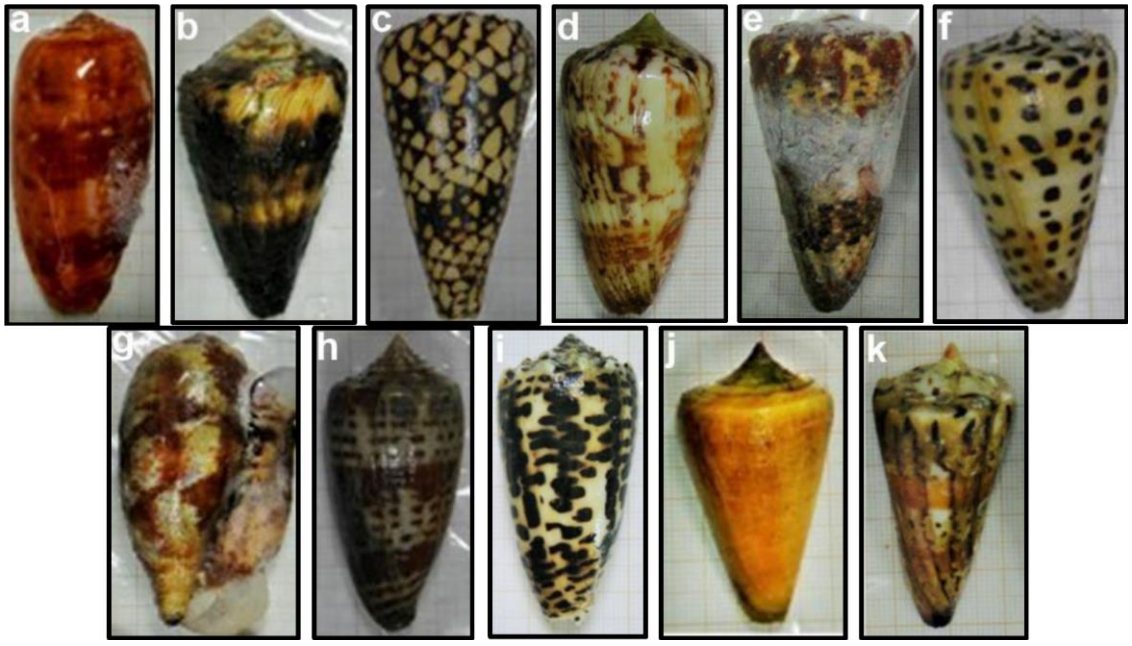

Figure 1. Photographs of 11 shells of cone snails collected from Nha Trang Bay, Vietnam, including *Conus betulinus* (a), *Conus miles* (b), *Conus bandanus* (c), *Conus caracteristicus* (d), *Conus eburneus* (e), *Conus imperialis* (f), *Conus tulipa* (g), *Conus lynceus* (h), *Conus pulicarius* (i), *Conus quercinus* (j) and *Conus generalis* (k)

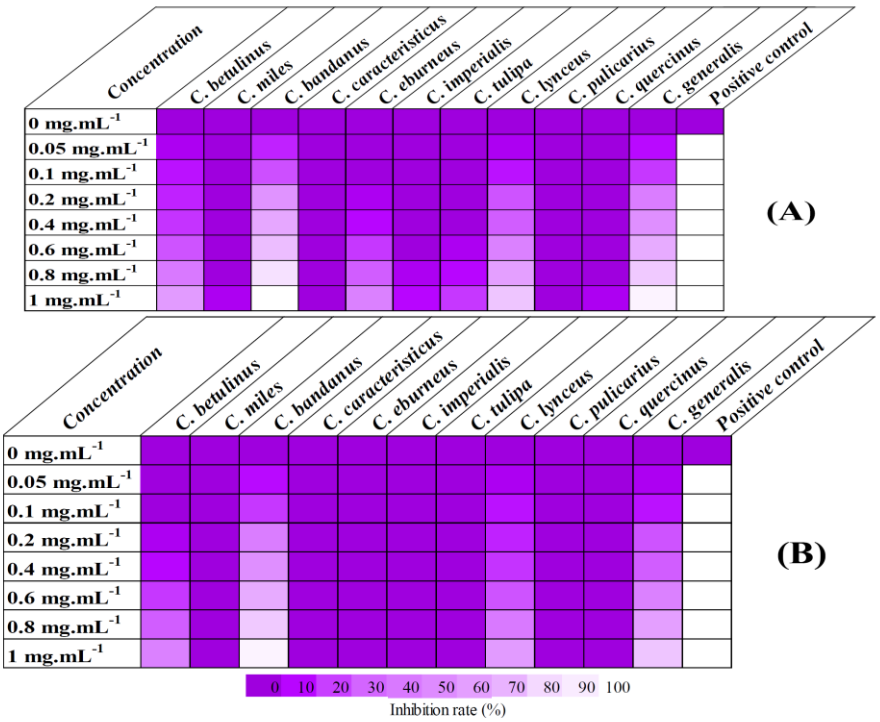

Figure 2. Toxicity of the venoms of the different *Conus* snails against *Anopheles stephensi* cell (A) and *Aedes aegypti* cell (B). Lighter purple colours indicate higher inhibition, while darker colours indicate lower inhibition

Toxicity assay screening for venoms that inhibited lethal effects on mosquito larvae

C. generalis venom was efficacious after 24 h which had 100% mortality (Fig. 3). It was noted that *C. bandanus* venom was more efficient, especially after 8h which had 100% mortality for *An. stephensi*. The remaining *Conus* extracts could have been more efficient after 24 h. Hence, these 5 *Conus* extracts were further investigated for the compounds responsible for activity using activity-guided separation and purification.

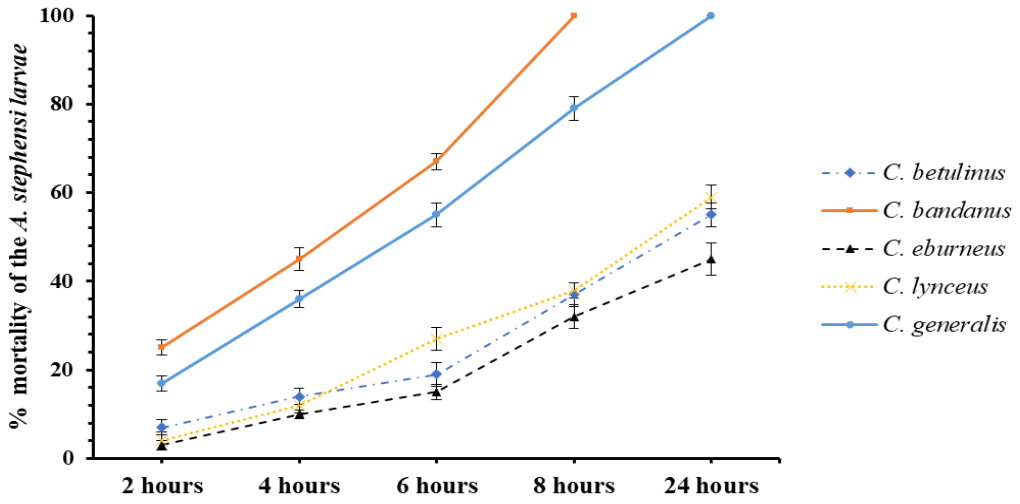

Figure 3. Larvicidal activities of different *Conus* crude venoms on *Anopheles stephensi*

Fractionation of insecticidal crude venoms by RP-HPLC

Figure 4 shows the HPLC profile for *C. bandanus* venom as a selective example. Fractionation was performed with 2–5 mg of crude venom and this work was repeated several times until each *Conus* venom was drained. The one-minute eluant fraction contained the peak, which was labelled with red marks, having significant inhibition on mosquitoes. These active fractions of *C. bandanus* venom were coded CB37, CB39, CB41, and CB47 by the time, respectively. Among them, CB41 fraction is a potential candidate, marked with a red asterisk. This fraction was collected between minutes 41–42.

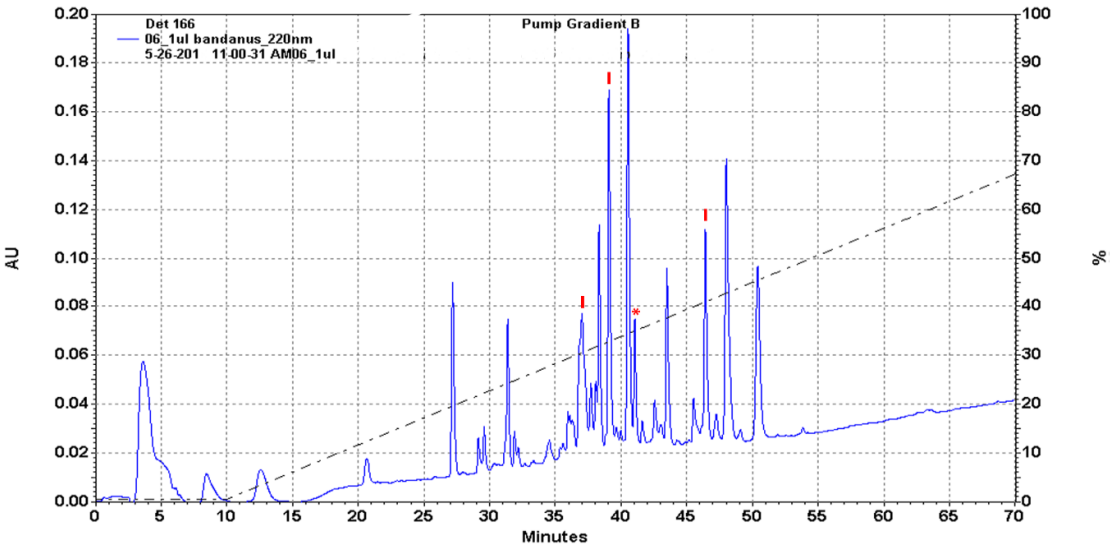

Figure 4. HPLC fractionation profiles of venoms of *Conus bandanus*. *Note:* the red lines and asterisk fractions showed significant and potential bioactivity on mosquitoes, respectively

MTT-based cytotoxicity assay screening for *Conus* venom fractions that inhibited cell survival

We carried out an MTT assay for fractions of each *Conus* venom (such as *C. betulinus*, *C. lynceus*, *C. eburneus*, *C. bandanus*, *C. generalis*). Most of the fractions had weak or unclear cytotoxicity when compared to the positive control (data not shown), except in the case of *C. bandanus* venom where a few fractions showed better toxicity (Fig. 5). Four of the fraction candidates (CB37, CB39, CB41, CB47 were marked red signs in Fig. 4) exhibited moderate toxicity to high toxicity as at least > 20% of cells died. The p-value was calculated for the effect of each *Conus* fraction versus the DMSO control. Due to their active effect on cell survival, these four candidate insecticides were analysed further.

Lethal effects of selected *Conus* fraction on adult mosquitoes

We further analysed the four *C. bandanus* fraction candidates and investigated their mosquitocidal activities to determine their potential as possible insecticides. These fractions displayed various mosquitocidal activities (Fig. 6). The toxicity of the CB41 fraction at 50 µM to mosquito adults was close to 75%, while the toxicity of the other three fractions (CB37, CB39, CB47) against mosquito adults was only < 25%. It is noteworthy that these fractions have highly hydrophobic properties. To our knowledge, hydrophobic peptides pose challenges in determining their structure, as well as their mechanism of action. Therefore, we prioritised the investigation of fraction CB41 with the clear anti-mosquito activity. We conducted a serial dilution of CB41 from

0 µM to 100 µM final concentration. We determined that the effect of CB41 was dose-dependent, and its LC_{50} was 30 µM (Fig. 7). Thus, we can conclude that extract CB41 is a potential *Conus* candidate for the control of *Ae. aegypti*, *An. stephensi* mosquitoes.

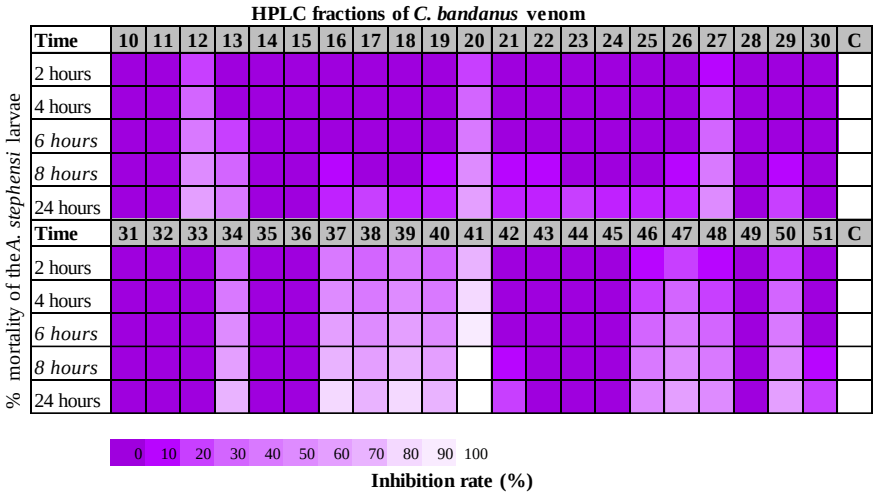

Figure 5. Toxicity of the different HPLC fractions of the *Conus bandanus* venom against *Anopheles stephensi* cell. Lighter purple colours indicate higher inhibition and darker purple colours indicate lower inhibition

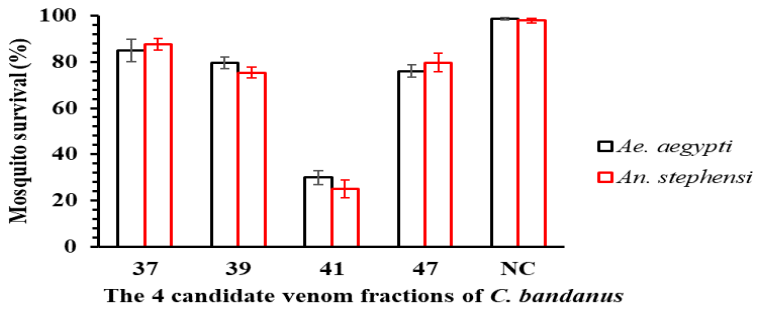

Figure 6. Survival rates of adult mosquitoes (*Aedes aegypti* and *Anopheles stephensi*) were treated with 50 µM of four candidate fractions. *Note:* NC: negative controls

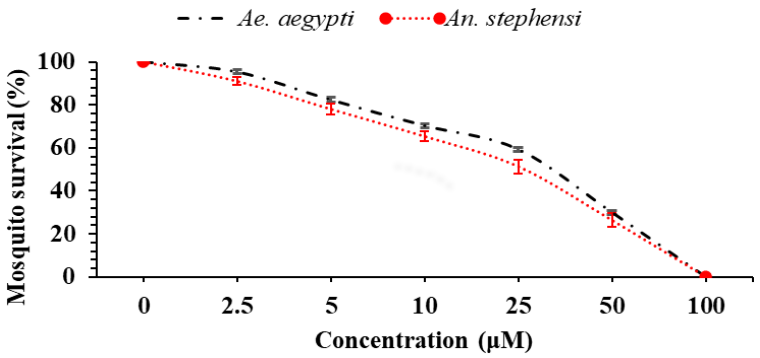

Figure 7. Dose-mortality curve of adult mosquitoes (*Aedes aegypti* and *Anopheles stephensi*) treated with CB41 fraction

Mass spectrometry analysis

Following the finding that CB41 fraction could effectively kill mosquitoes, we investigated further its purity. Based on the chromatogram profile (Fig. 8), the purity of the CB41 fraction was acceptable (ranging from 70% to 85%).

The CB41 fraction was submitted to mass analysis. Thus, MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) MS analysis of the native CB41 conopeptide in *C. bandanus* venom showed a $[M+H]^+$ species, detected at *m/z* 3333.1 that characterized a toxin molecular mass of 3332.1 Da (Fig. 9). Following the total reduction with TCEP, the $[M+H]^+$ species was detected at *m/z* 3339.22, which indicated the presence of three disulphide bonds in CB41 (net increase of 1 Da for each cysteine present). Thus, the CB41 fraction was *C. bandanus* conopeptide possessing molecular weight 3332.1 Da, with three disulphide bonds.

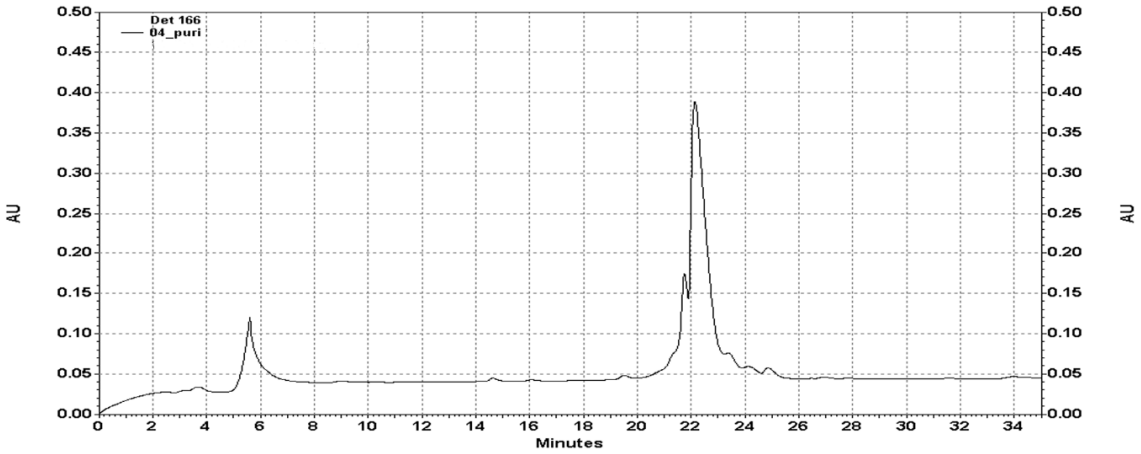

Figure 8. HPLC purification profile of mosquitocidal fraction (41st min peak)

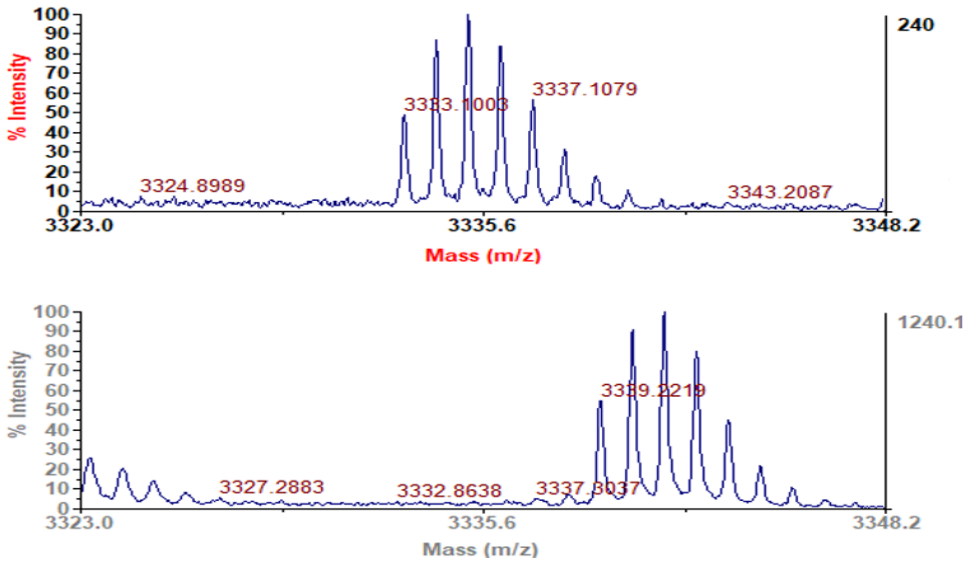

Figure 9. Determination of the cysteine number in native conopeptide (upper graph) and on its reduced form by TCEP (lower graph) from MALDI-TOF MS. Note the shift of 6 Da characterising the reduction of three disulphide bonds

DISCUSSION

Peptides are valuable alternative to chemical pesticides for insect control. A reported example of an insecticidal *Conus* peptide was the alpha-ImI from *Conus imperialis* (Yu et al., 2012). Conopeptides can specifically bind to nicotinic acetylcholine receptors, neurotransmitter transporters and all types of ion channels in the insect nerve cell; weak toxicity to mammals, however, can potently poison insects.

In our preliminary study, we collected and screened venom fractions of 11 different *Conus* species. We have identified one conopeptide of *C. bandanus*, named CB41, as a potential candidate for mosquito control. The CB41 conopeptide, to our knowledge for the first time, can kill both adult mosquitoes (*An. stephensi*, and *Ae. aegypti*). The LC_{50} of this conopepide for the adult mosquitoes was about 30 μM, which is lower than 1.6–2,300 times that of oostatic peptides, which influence mosquito reproduction because of regulation of the eggs' development (Hlaváček, 2013). Otherwise, most Vietnamese fishermen do not know that these *Conus* species have dangerous venom glands, and also its research significance in medicine (Rigo et al., 2013), pharmacology (Lewis et al., 2012) and agriculture (Gao et al., 2017). These snails are occasionally harvested and sold for food. That causes the number and species of this snail species in Vietnam to decline very seriously. It is very necessary to preserve this *Conus* snail species and artificially cultivate it in the future.

We also initially determined the molecular weight of the CB41 peptide, which was 3332.1 Da and three disulphide bridges. This peptide has been estimated theoretically to have approximately 31–35 amino acid residues. It possessed a long sequence, hydrophobic properties, and compact structure, which are different oostatic peptides (proven a decapeptide), and ImI conotoxin (short peptidic sequence with two disulphide bridges). CB41 length was difficult to determine its primary sequence by only de novo peptide sequencing mass spectrometry. We have found no clues to sequence this long peptide, nor the short sequences of those small fragments (data not shown). It could be possible that this conopeptide could contain some amino acids, such as proline or hydroxyproline, and its smaller fragments containing these residues have very strong signals. This could be obscured by other mass spectrum signals. Our case study is similar to the previously reported BtX conotoxin (Fan et al., 2003), in that many modern analytical methods are needed to solve its primary structure. Thus, the CB41 primary structure may be determined by using a combination of analytical methods such as Edman's degradation, enzyme hydrolysis combined with mass spectrometry and/or transcriptome.

CONCLUSION

In this study, we collected 11 *Conus* species in Nha Trang Bay. Through MTT assay and toxicity assay on mosquito larvae, we have screened five of eleven crude venoms that inhibited cell survival and mosquito larvae. To investigate further, we fractionated minutely these five crude venoms and tested them for MTT cytotoxic activity. As a result, we found four fractions in *C. bandanus* venom that have cytotoxic activity against cell survival, named CB37, CB39, CB41, and CB43, respectively. We performed toxicity experiments on adult mosquitoes with these fractions and found that the CB41 fraction was a potential candidate, with IC_{50} = 30 μM. We have also determined that this conopeptide possesses a molecular weight of 3332.1 Da and three disulphide bridges. Through initial results, the determination of its structure may be a challenge.

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