RESEARCH ARTICLE

Phlogiellus bundokalbo spider venom: Its neuroactive, phospholipase a2 and cytotoxic components against human breast adenocarcinoma (MCF-7)

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ABSTRACT

Spider venoms and toxins are valuable sources of lead compounds for drug development due to their essential role in cellular and physiological processes targeting various receptors. Here, we present the protein profile of the venom of *Phlogiellus bundokalbo*, an endemic Philippine tarantula, to screen and characterize its cytotoxicity against MCF-7 cells, secretory phospholipase a2 (sPLA2), and neurotoxicity to evaluate its potential anticancer properties. Spider venom was extracted via electrical stimulation. Venom components were fractionated by reversed-phase high-performance liquid chromatography and characterized through liquid chromatography-mass spectrometry (LC-MS) and SDS-PAGE analysis before assay. The resulting five venom fractions were amphiphilic peptides showing cytotoxicity against MCF-7 cells in a concentration-dependent manner (IC50 ranging from 52.25µg/ml to 110.20µg/ml) after 24-hour incubation. Cells appeared detached, rounded, and shrunk with cytoplasmic condensation upon overnight incubation with venom fractions. The sPLA2 was observed in all the venom fractions tested for cytotoxicity. Venom fractions revealed a predominant mass of ~3-5 kDa with LC-MS analysis. Results showed distinct similar mass as µ-theraphotoxin-Phlo1a, an Australian tarantula, *Phlogiellus* sp. toxin with inhibitor cystine knot motif. The venom fractions exhibit excitatory neurotoxins that might activate presynaptic voltage-gated ion channels, such as an agonist or gating modifier toxins that slow down the channel inactivation similar to spider toxins. In conclusion, the spider venom of *P. bundokalbo* exhibits cytotoxic, phospholipase A2, and neuroactive properties suggesting that its venom components, upon further purification and structure-function analysis, can be potential tools in the development of targeted breast chemotherapeutics.

Keywords: *Phlogiellus bundokalbo; spider venom; cytotoxicity; MCF-7; phospholipase A2; neuroactive*

Introduction

Breast cancer is the most common cancer among women that affects 2.1 million women each year and leading cause of cancer-related deaths among women worldwide [1,2]. In the Philippines, it is the most frequent cancer among men and women, excluding non-melanoma skin cancers. Depending on the kind of breast cancer and how far it has spread, the current gold standard of care for cancer is a combination of surgery, chemotherapy, hormonal therapy, biological therapy, or radiation therapy [3]. However, certain drawbacks such as nonselective distribution of drugs, multidrug resistance, amplified drug toxicity, adverse side effects to discriminate between cancerous and normal cells are associated with the treatment [4]. Thus, there is a great need to continuously explore novel, safe, efficient, and selective anticancer agents that can be used alone or in combination with the current U.S. Food Drug Administration (FDA) approved drugs for breast cancer prevention and treatment.

MCF-7 (Michigan Cancer Foundation), a human breast cancer cell line derived from a metastatic mammary

carcinoma, is an ideal *in vitro* model for breast cancer studies due to its characteristic differentiated mammary epithelium and its distinct estrogen, progesterone, and glucocorticoid receptors in the cytoplasm [5,6]. Due to its sensitivity to several hormone receptors, MCF-7 is often used to analyze anticancer agents in breast tumor heterogeneity.

In the last five years (2015-2020), the U.S. FDA has approved peptides or peptide-containing molecules, which account for 7% of the total number of therapeutics in the market [7]. In this context, venom-derived therapeutics have shown great potential to diverse pharmacological and neurobiological applications with currently eleven (11) U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approved toxin-based molecules available in the market from animal sources such as cone snails, lizards, leeches, snakes and spiders while several more venom peptides are already currently in clinical trials or preclinical development phase [8].

Spider venom, being one of the subject of interest for potential therapeutic peptides, is composed of diverse and complex mixtures of low molecular weight compounds, peptides, amino acids, and polyacylamines. These natural concoction of potentially therapeutic molecules have been previously studied to contain molecules that can prevent stroke-induced brain damage, Alzheimer's disease, epilepsy, cardiovascular problems and cancer which have high specificity, selectivity and stability towards proteins and protein sub-types [9-12]. They are valuable candidates of novel cancer chemotherapeutics due to their versatile proteins and peptides which include enzymes (such as proteases, hyaluronidases, and phospholipases), neurotoxins (most have disulfide-rich peptides affecting ion channels), and cytolytic peptides which can induce cell death. Consequently, spider venom toxins exhibit potent cytotoxic properties in several cancer cells due to their selectivity in specific cancer subtypes and membrane ion channels such as voltage-gated channels, sodium, potassium, calcium, chloride channels, and transient receptor potential (TRP) channels, inhibition of enzymatic activities and cell cycle alteration and induction of apoptosis [12,13]. The majority of patents for cancer treatment use from spider venom and its derivatives are mainly peptides, phospholipase, and hyaluronidase sourced from *Loxosceles* sp. [14,15]. However, detailed description of the mode of action of the spider venom and its derivatives is yet to be explored.

Secretory phospholipase A2 (sPLA2) present in the venom plays an essential role in mediating various cellular processes, including cell proliferation, differentiation, apoptosis, and inflammatory response in various cancer cells such as breast, lung, and prostate [16,17]. For example, secretory phospholipase A2 group IIa(sPLA2-IIa) is used in liposomal drug delivery of chemotherapy and as a prognostic marker [17]. Moreso, the implication of sPLA2 stimulating the release of elevated levels of excitatory amino acids (EAA) is attributed to the pathogenesis of neural injury and death in central nervous system trauma [18,19]. The mechanism of sPLA2 action on EAA release remains unclear though it is suggested that PLA2 disrupts an artificial planar lipid bilayer in a Ca^{2+}-dependent manner [20]. Reports regarding phospholipases from spiders in cancer studies remain nascent. Studies with other animal species, the majority from snakes mainly due to its abundant venom supply, validates the antiproliferative activity of venom phospholipases in cancer studies. However, phospholipases from spider venoms in cancer studies (*in vivo* and *in vitro*) remain incipient.

To date, there are 1576 toxins curated in the Arachnoserver, a manually curated database containing information on the sequence, three-dimensional structure, and biological activity of spider toxins, of which the largest number of toxins were isolated from spiders of the family Theraphosidae [22]. Given that >99% of venom peptides are pharmacologically uncharacterized and most known toxins target ion channels, spider venom peptides remain a virtually untapped source of novel targets for therapeutic leads [23,24]. *Phlogiellus bundokalbo* is one of the five known endemic tarantula species in the Philippines and was first described by Barrion and Litsinger (1995) in Siniloan, Luzon Island, Philippines [21]. This study was designed to screen and characterize the venom components of *P. bundokalbo* for anticancer properties. Here, venom peptide fractions were isolated to determine its cytotoxicity against breast adenocarcinoma, MCF-7 cells, and its interaction with sPLA2in growth and proliferation of MCF-7 cells in vitro with excitatory neurotoxicity against *Tenebrio molitor* in vivo. With phlotoxin, PhlTx1, as the only known toxin isolated from *Phlogiellus* sp., *Theraphosidae Selenocosmiinae*, its use as antinociceptive agent against pain channel, Nav 1.7 have been studied [10]. Since the anti-cancer properties of the venom and its components of the venom of the Philippine endemic *P. bundokalbo* are just emerging, our findings complement important implications for the potential use of this spider's venom components in the development of potential breast cancer chemotherapeutics.

Methodology

Spider sampling and venom collection

Ten spiders were collected from Brgy. Spring, Kibawe in Bukidnon, Philippines, during the wet season of November

2017 by the team from Mindanao State University- Iligan Institute of Technology (MSU-IIT). It is an agroecosystem situated on a slope, tunnels, and underneath limestone rocks.

Before sampling, wildlife gratuitous permit R10 2017-35 was secured from the Department of Environment and Natural Resources (DENR) Region 10. Spiders were collected by sweep net and vial tapping using wide-mouth polyethylene plastic caps with small holes to allow air circulation inside the container [25,26]. Samples that will be used for identification were preserved in 80% ethyl alcohol. Initial examination of the collected spiders was done in MSU-IIT and was later on identified as *Phlogiellus bundokalbo* after further examination at the Museum of Natural History at the University of the Philippines in Los Baños, Laguna. Live spiders separated by enclosures were transported and reared at the University of Santo Tomas Research Center for Natural and Applied Sciences (UST-RCNAS) for venom collection. They were fed once in every two weeks with *Lateralis* sp. cockroaches, while maintaining the moisture in each enclosure by spraying with distilled water [25,26].

Spider venom extraction

Venom extraction was done by placing the spiders in a plastic container and was lightly anesthetized using carbon dioxide gas (CO_2) for five minutes. Once anesthetized, the spider's fangs were positioned in a 1.5mL tube to collect the venom discharge at the fang's tip and avoid contamination with digestive fluids. Before electrical stimulation, the fangs were washed with a minute amount of distilled water. A pair of stimulator electrodes were then placed in contact with the fissure between the chelicerae and cephalothorax to ease the electrical current transmission. The electrical stimulus of 10V was applied for two seconds with a three-second interval between shocks for one minute. After extraction, the venom was centrifuged, freeze-dried, and stored at -20°C until used. The dry weight of each venom sample was determined using an analytical balance. The spiders were given food and water a day after the procedure.

Out of the ten tarantulas, five (5) were collected for venom extraction while the rest were used for morphological and identification purposes. Venom extraction was performed twice a month to give the tarantula sufficient time to replenish its venom supply. Collected venom samples for a specific month are pooled before assay.

Direct purification of crude venom by HPLC chromatography

In order to separate the components of the venom for assays and further characterization, crude *P. bundokalbo* venom was subjected to HPLC using a Waters E265 HPLC system (Massachusetts, USA on an Agilent Eclipse Plus C18 column (5um, 4.6 x 150mm) at a flow rate of 1 ml/min, using a two-component mobile phase system in which mobile phase A is 0.1 % trifluoroacetic acid (TFA) in water (EMD, USA) and mobile phase B is acetonitrile (Spectrum, New Jersey) and 0.1 % TFA in water. Here, 100 ul of 0.6 mg of lyophilized crude venom dissolved in 1 ml of HPLC water, treated with 10% acetonitrile, 2% TFA was injected in the column and initially run at a linear gradient of 5-95% solvent B in 100 minutes at a flow rate of 1ml/min [27]. The following gradient was employed to separate the amphiphilic peptides further: 5-35% acetonitrile in 18 mins, 35-46% acetonitrile in 12 mins, 46 to 100% in 20 minutes, 100 % acetonitrile for 5 mins, then 100-5% acetonitrile in 4 mins. The peaks were monitored at 220 nm absorbance and peak fractions collected manually, identified, lyophilized, and dry weight of each sample was determined using an analytical balance.

Characterization of toxin fractions by SDS-PAGE

Following the HPLC purification, the purity and approximate molecular mass of toxin fractions were determined using sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) following the established Laemmli with some modifications. The percentage of resolving gel was made to 12.5%; then samples were run at 130 Volts for ~30 minutes to allow the separation of medium to low molecular weight components of toxin fractions. As a standard, unstained low molecular weight protein marker (Thermofisher Scientific, Illinois, USA) was used as the basis for approximating the molecular mass while 2x SDS sample buffer (CSH Protocols) was used to track the migration of the fractions in the gel during the run. For detection, the gel's staining was done by using the solution containing approximately 0.2 grams of Coomassie brilliant blue R-250 dissolved in 50% methanol, 40% water, and 10% glacial acetic acid.

Characterization of crude venom through LC-MS analysis

The lyophilized crude *P. bundokalbo* venom was analyzed via on-line LC-MS using a Waters H-class UPLC-QTOF with a Waters Acquity CSH C18 1µ 2.1 x 50 mm column. Solvents A (0.1% formic acid in distilled water) and solvent B (0.1% formic acid in acetonitrile) were used at a flow rate of 0.3 mL/min. Peptides elution started from 5-30% of B in 2 min, followed by a second one from 30% to 50% in 2.5 min, then from 50% to 95% in 3.5 min and finally a last step from 95% to 100% in 5 min. This gradient was similar to the HPLC method used to better separate the venom components in

the amphiphilic region, leading to more peptides detected and fragmented than classical gradients. MS parameters were as follows: Sampling Cone Voltage 40.00 V, Capillary Voltage 2.8 kV, Source Temperature 120 deg C, Desolvation Temperature 450 deg C, Desolvation Gas Flow 800 L/hr. Scans were done for 8.00 minutes over an m/z range of 400.00 - 2000.0 with 0.500 sec scan intervals with a leucine enkephalin lockspray reference. Data were acquired and processed using MassLynx 4.1 SCN 937 software and exported with mMass software ver 5.5.0 for analysis [28,29].

Functional characterization of spider venom fractions

Cytotoxicity assay using MTT assay

The crude and venom fractions were functionally characterized for cell viability using MTT assay (Promega, Madison, USA) according to manufacturer's protocol. This assay is based on the cleavage of MTT that forms formazan crystals. Before assay, lyophilized venom fractions are weighed in an analytical balance. Different concentrations (25, 50, 100, 200 µg/ml) of the samples were prepared by dissolving in a specified cell culture medium; the culture medium was used as the negative control.

MCF-7 (American Type Culture Collection, ATCC) were cultured in T25 flasks (Corning, Tewksbury, MA, USA) using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with F12 Ham, 10% fetal bovine serum (FBS), and 1% antibiotic-antimycotic. All reagents and media used in this assay are from Sigma-Aldrich, Singapore. Cells were maintained in an incubator at 37°C with 5% CO_2 supply and 95% humidity. Confluent cells (80%) were used in the experiment. The cells were detached using 5% trypsin-EDTA and were transferred in a sterile conical tube, centrifuged for five minutes at 3000rpm. The cells were washed with sterile phosphate buffer saline (PBS; pH 7.4) twice. Then, cells were subcultured in a 96-well plate at a density of $1x10^4$ cells/well. After 24-hr of incubation, the medium was replaced with 100µL of DMEM containing different venom fractions concentrations. After a 24-hour incubation period, the cells were washed twice, and 50µL of sodium 3'-[1-phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene-sulfonic acid hydrate (MTT) at 1mg/mL concentration was introduced. The cells were incubated for 4 hours before adding 100 µL dimethyl sulfoxide, and absorbance was read at 570nm using Multiskan GO Microplate Spectrophotometer (Thermo Scientific, USA). Average of four readings and blanks were utilized to determine the inhibition rate. Absolute IC_{50} values, concentrations producing 50% inhibition, were determined by plotting the percent mean activity over the log of concentration in the nonlinear regression curve fit function (log of inhibitor against four parameters variable slope response, and least-squares fitting method with interpolation at 95% confidence interval) of the Graph Pad Prism 6 software.

Assessment of Secretory Phospholipase A2 activity (sPLA2)

The sPLA2 of the venom fractions were evaluated via colorimetric assay with L-a-lecithin as a substrate. All reagents and standard PLA2 from bee venom used in the assay was from Sigma-Aldrich, USA following manufacturer's protocol [32]. The standard assay mixture contained varying amounts of Tris-HCl buffer, L-Deoxycholate, 1% Lecithin, and $CaCl_2$ were mixed and incubated for five minutes at 37ºC.

In a 96-well plate, 10 µL of Tris-HCl buffer, PLA2, and spider venom samples were added in the standard, and experimental wells. Ten microliters of venom fractions and the same volume of standard PLA2 from bee venom were used. Using the reagent mixture prepared, 40 µL of it was added in each well and was incubated for 5 minutes at 37ºC. After incubation, 180 µL ethanol/ether, 15 µL Hydroxylamine-HCl, and 15 µL NaOH were subsequently added in each well and incubated for 20 minutes at room temperature. Afterward, 30 µL of 3N HCl and FeCl3 was added in each well. All the standards and the samples were read at 570 nm using the Multiskan GO Microplate Spectrophotometer (Thermo Scientific, USA). A standard curve was utilized with different concentrations of lecithin and $CaCl_2$ to evaluate sPLA2 activity. Each test was performed in triplicates.

Behavior responses of T. molitor treated with spider venom fractions

Tenebrio molitor, commonly known as mealworms, between 3rd and 4th instar (~150 mg/individual) were used. For each mealworm, 2.0 µL of toxin diluted in ultrapure water was injected into the metathoracic pleurite. Injections were performed using a 29.5 gauge insulin syringe (B–D Ultra-Fine, Terumo Medical Corporation, Elkton, MD). Three replicates of ten (10) mealworms were used for each venom fraction, and the same number of control insects were injected with ultrapure water and maintained under the same conditions. The fractions were observed at 5, 30, and 60 min intervals to record phenotypic responses. A numeric score was assigned to each state and averaged to indicate the effect of the toxin previously described by Hardy *et al*. (2013) [29].

Results

Since *P. bundokalbo* was first described in 1995, it had undergone various taxonomic revisions to resolve any

synonymy conflict within its genus, the latest of which is by Nunn *et al*. (2016)[38]. Previous site location records in this Philippine endemic tarantula include: Siniloan, Laguna, and Real, Quezon Province, found on upland rice field near secondary forests, both of which are located on the Island of Luzon wherein occurrence was first recorded [25,26]. The collection site (Figure 1A) is described as agroecosystem environment near a riparian area.

The genus *Phlogiellus* is described as a small to medium-sized theraphosid (Figure 1B-C). It is described by the absence of any stridulating organ and presence of undivided tarsal scopulae on legs I in the male and I-IV in the female, foveal groove almost straight, all tarsal scopula undivided [21]. They construct their nests or shelters with one or two silk-lined openings used for detecting prey movement via vibration [26].

After electrical stimulation, venom was expelled from the spider's chelicerae. Its venom yields 10-20 µL of clear and colorless liquid that is soluble in water. Its venom yields 10-20 µL of clear and colorless liquid that is soluble in water showing a homogenous distribution of hydrophobic and hydrophilic compounds eluting from 0% to 70% acetonitrile (Figure 2A). Its venom showed a higher concentration of compounds eluting between 20% to 35%, indicating a moderate hydrophilic nature of the venom. After 50 minutes, no more fractions were found eluting in the venom. Out of seven venom fractions, only five venom fractions were run in the gel and used to evaluate functional activity to focus on the amphiphilic peptides present in the venom with anticancer properties.

Following the purification step of the crude venom, the purity and the estimated molecular mass of the collected

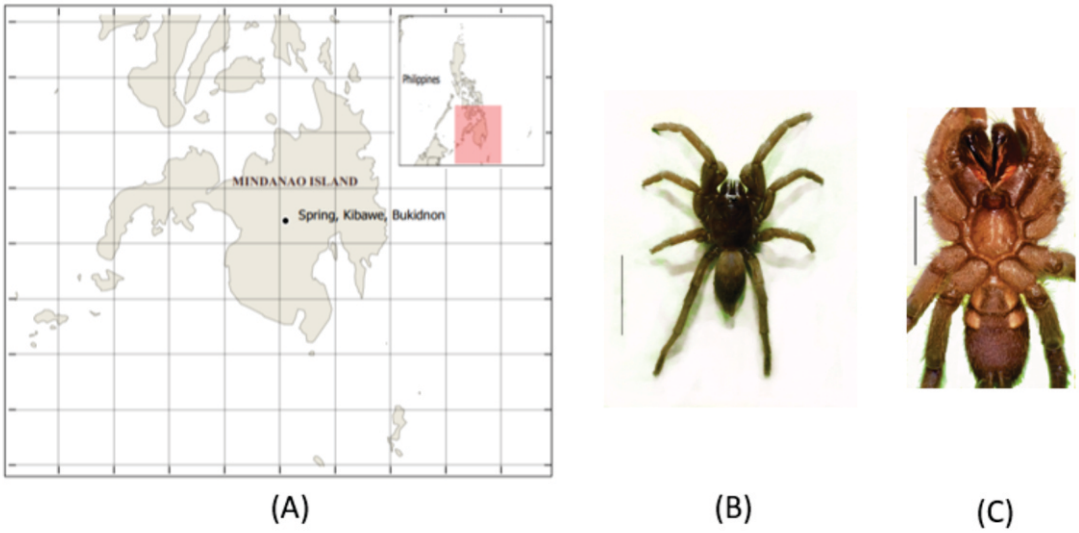

Figure 1. *Collection site in Kibawe, Bukidnon (A), dorsal (B), and ventral (C) view of P. bundokalbo. Scale bar of A is 10mm, and B is 4mm.*

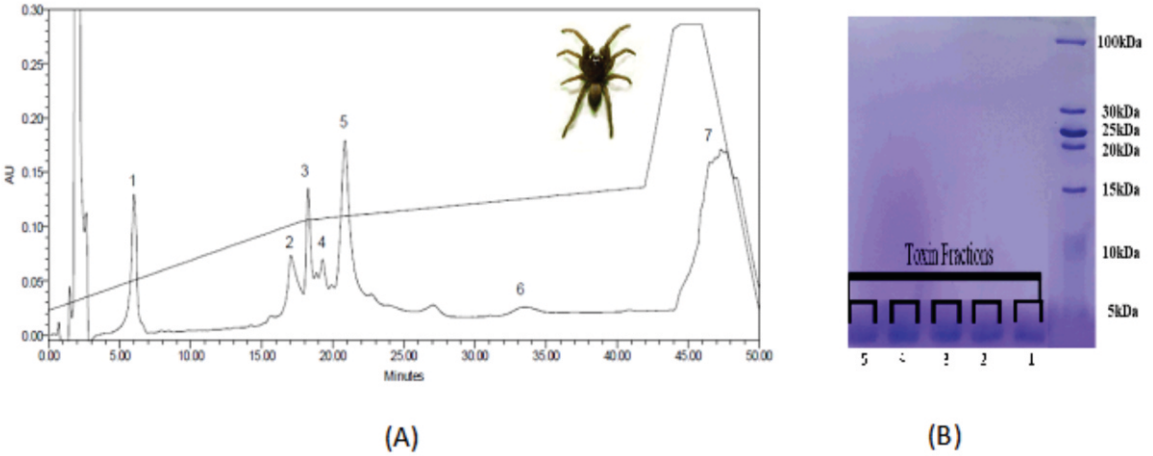

Figure 2. *Chromatographic profile of crude P. bundokalbo venom using C18 column: absorbance was measured at 220nm (A). Fractions are numbered as manually collected from HPLC. Toxin fractions collected from HPLC purification were subjected to SDS-PAGE using a 12.5% resolving gel (B). Out of seven venom fractions shown in the gel, only five were used to evaluate the amphiphilic peptides' functional activity.*

fractions were assessed using SDS-PAGE (Figure 2B) with each toxin fraction displaying one clean band, suggesting either the purity of the sample or the uniformity in the identity of the components in each fraction. Also, all bands of spider venom fractions displayed a peptide mass of <5.0 kDa. Additionally, the crude spider venom generated 135 peptides of masses ranging from 3-9 kDa, most of which are ~3-5 kDa after being subjected to LC-MS (Figure 3A and 3B).

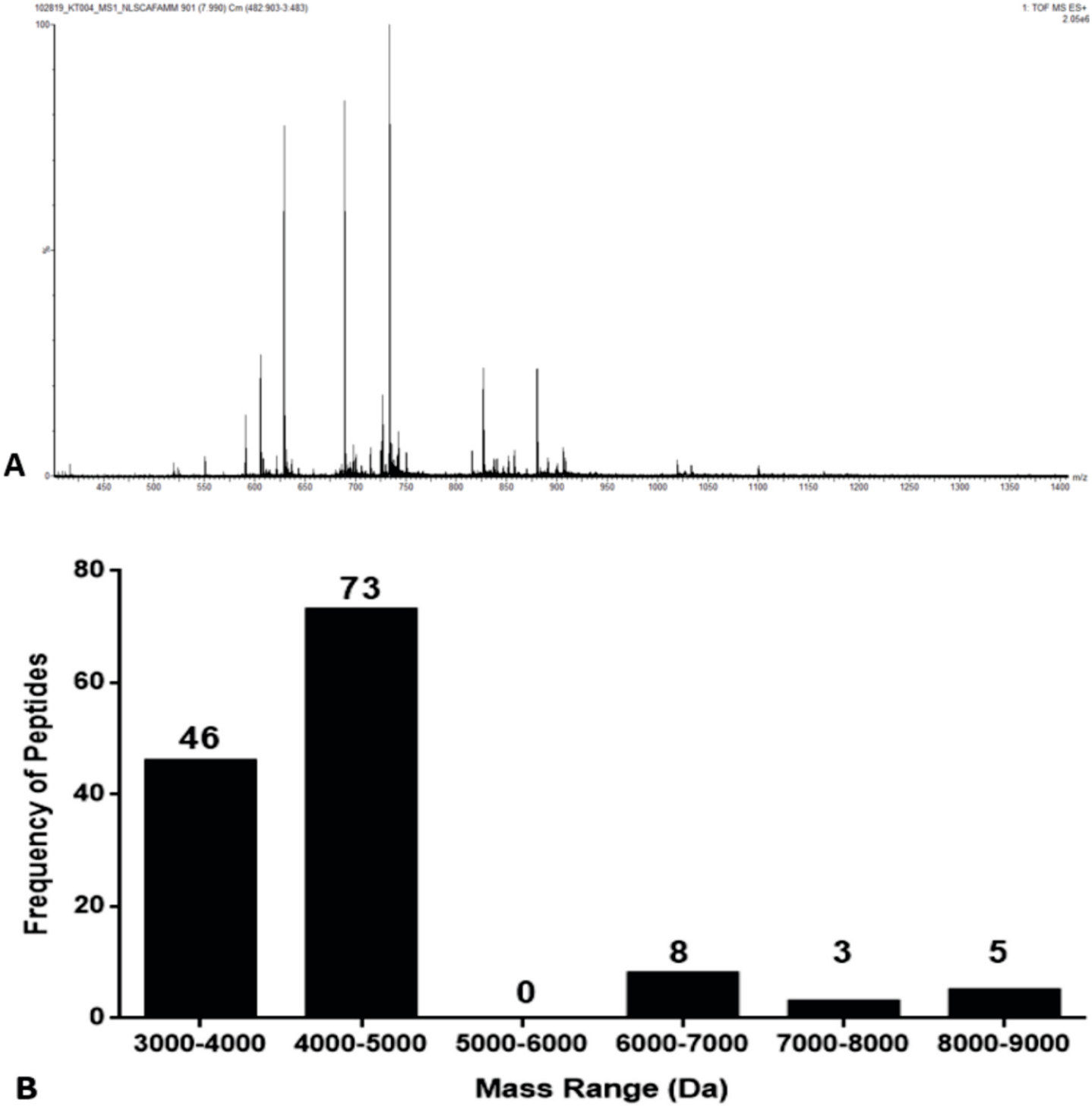

Figure 3. *LC-MS spectra of P. bundokalbo crude venom (A). Scans were done for 8.00 minutes over an m/z range of 400.00 - 2000.0 with 0.500 sec scan intervals with a leucine enkephalin lockspray reference. Data were acquired and processed using MassLynx 4.1 SCN 937 software and exported with mMass software ver 5.5.0 for analysis. Peptide mass distribution generated from the LC MS of the crude venom showing 135 peptides of mass range 3-9 kDa (B)*

Cell viability using MTT assay was used to evaluate the effect of different concentrations of venom fractions on mitochondrial functions based on the capability of the mitochondria of a living cell to reduce MTT dye (yellow color) into a formazan crystal product (blue to purple coloration) of which absorbance was read at 570nm. A significant cytotoxic effect of venom fractions in the growth and proliferation of MCF-7 cells was observed at 25, 50, 100, and 200 µg/ml ($p<0.05$). The cytotoxic effect is concentration-dependent. IC50 values of venom fractions range from 52.25 µg/ml to 110.20µg/ml after 24-hour incubation. Due to significantly reduced cell viability in concentrations of 25 and 50µg/ml in venom fraction 3 ($p<0.05$), morphological analysis was evaluated using inverted light microscope.

Morphological analysis of the MCF-7 cells treated with 25 and 50 µg/ml of spider venom fraction 3 was further evaluated after 24-hour incubation. Comparing the results of untreated MCF-7 cells, which initially showed homogenous dispersion with distinct boundaries (Figure 5A), cell number has reduced from 25 to 50µg/ml after 24 hours incubation of venom fraction 3 (Figure 5B and 5C). Cells appeared detached, rounded and undergone cytoplasmic condensation and cell shrinkage at 50µg/ml of venom fraction 3 (Figure 5C). At 25 µg/ml of venom fraction 3, cell shrinkage and cytoplasmic condensation were also noted, but cells were observed to increase (Figure 5B).

Considering the predominant protein composition (392 g protein/mg of crude venom) in the *P. bundokalbo* venom, the proteins' enzymatic activity, mainly for PLA2 and proteolytic activity, was tested in five fractions (Figure 6). All the five venom fractions showed low enzymatic activity compared to the enzymatically active PLA2 from bee venom

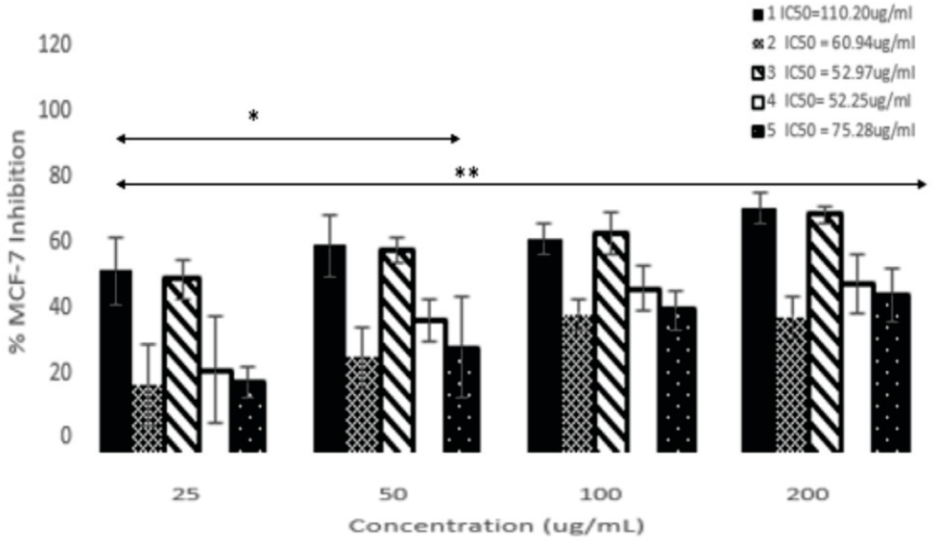

Figure 4. *Concentration-dependent inhibition of MCF-7 cells by P. bundokalbo venom fractions after a 24 hour incubation period. Averaged data from three trials with triplicate readings are shown. **Statistically significant interaction between the effects of venom fractions and different concentrations in percent inhibition of MCF 7 cells; *Significantly difference in percent inhibition between concentrations 25 and 50µg/ml, $p<0.05$.*

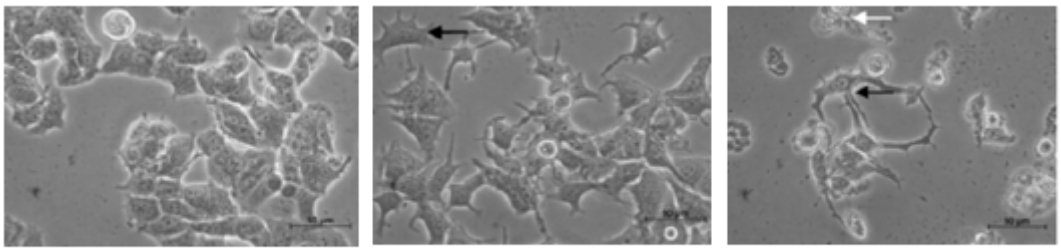

Figure 5. *Morphological changes of the effects of the venom of P. bundokalbo on MCF-7 cells monitored for 24 h. Representative images of the untreated MCF-7 cells (A) showing homogenously dispersed with distinct boundaries after overnight incubation. After treatment of the cells with 25 µg/ml of venom fraction 3 (B), MCF-7 cells showed cell shrinkage and rounding (black arrow). Cells appeared to detach from the substratum (white arrow) after treating the same venom fraction with 50µg/ml (C). Increasing concentration of venom fraction showed a decrease in cell number (B). Magnification: 400x; scale bars represent 40um.*

indicating the presence of PLA2 in these venom fractions, with venom fraction 4 having the least PLA2 activity ($p<0.05$). Venom fraction 5 showed the highest PLA2 activity level, similar to fraction 3 ($p<0.05$).

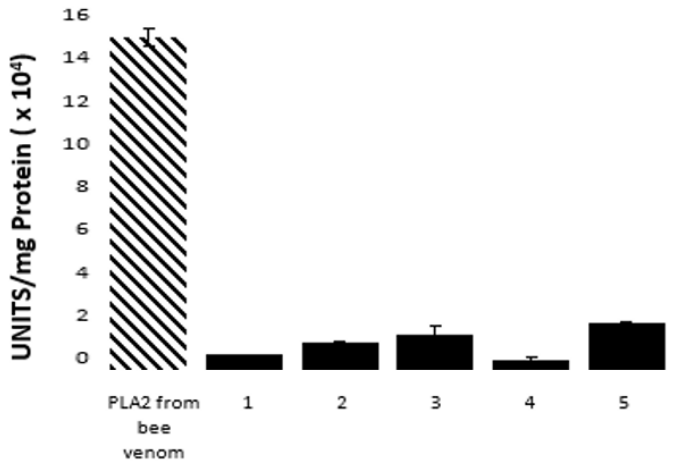

Figure 6. *The sPLA2 activity of venom fractions from P. bundokalbo vs. standard PLA2 from bee venom. Numbers represent venom fractions used. Results are averaged value from three trials with triplicate readings. PLA2 activity was significantly lower in the venom fractions than the standard bee venom (1.39 x 10-3 ± 5.77x10-6 units/mg, p<0.05). Venom fraction 4 has the least sPLA2 activity whereas venom fraction 5 showed the highest sPLA2 activity, similar to fraction 3 (p<0.05).*

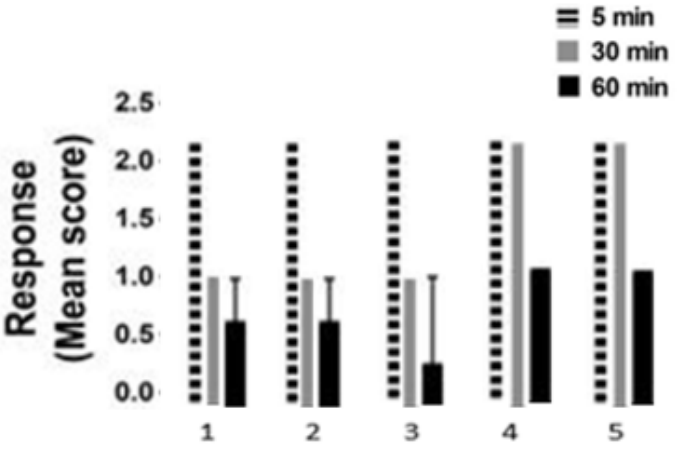

Figure 7. *Phenotypic response of mealworms, T. molitor, with spider venom and monitored at 5, 30 and 60 minutes. The response was scored as 2, 1, 0, -1, and -2, respectively. (Note: a score of 2 exhibits excitatory movement more erratic than control insect; 1 exhibits excitatory paralysis and not able to move independently; 0 is dead or moribund; -1 exhibits depressive paralysis showing the insect is effectively paralyzed; -2 exhibits depressive movement. The spider venom fractions exhibited scores of 0.5-2.0 indicating excitatory response. No dose produced a depressed state of any of the time points. Columns represent mean± SEM of three replicates of ten (10) mealworms for each dose.*

As seen in Figure 7, a concentration-response curve was shown to quantify the phenotypic response of the spider venom fractions based on observed states of behavioral response of *T. molitor* when injected with venom fractions vs. water as control after 5, 30, and 60 minutes in order. This was done to determine whether the spider venom fraction was excitatory or depressive [29]. Interestingly, the spider venom fractions exhibited scores of 0.5-2.0 indicating excitatory responses.

Discussion

Spider venoms are rich sources of low molecular weight compounds, peptides, amino acids, and polyacylamines with diverse pharmacological properties. Spider venom's therapeutic potential can be attributed to its bioactive components that can affect the physiological activities of animals or human exposed to it [30]. Spider venom peptides have been identified as anticancer drug candidates due to its interaction with the cancer cell membrane, membrane phospholipids, and ion channels primarily used by spiders for rapid prey immobilization and defense [12,33,34]. In the present study, the protein profile of the venom of *P. bundokalbo* is screened and characterized for its cytotoxicity against MCF-7 cells, sPLA2 activity, and in vivo neurotoxicity to evaluate its anticancer properties.

The venom of *P. bundokalbo* contains a diverse mixture of components which orchestrates the full toxicity of the venom. Its venom showed a higher concentration of compounds eluting between 20% to 35%, indicating a moderate hydrophilic nature of the venom. According to the UV spectra library, peak fractions detected in the chromatogram at 220 nm correspond to peptide bond detection [35]. Results in Figure 2A showed venom fraction 1 corresponds to low molecular weight compounds in the polar region, venom fractions 2-5 constitute the low molecular weight peptides in the semi-polar region whereas venom fractions 6-7 constitute high molecular weight compounds [23,36]. Venom fractions 1-5 were selected to confirm the presence of low molecular weight peptides (<5kDa) based on the SDS-PAGE profile (Figure 2B) and further evaluated to test for anticancer properties due to its amphiphilic nature.

As seen in the LC-MS profile of the crude venom of *P. bundokalbo* (Figure 3A and 3B), its venom components contain polar low molecular compounds, semi-polar peptides (~2-8 kDa), and nonpolar proteins and enzymes greater than 8kDa (Escoubas *et al*., 2000; Klint *et al*., 2012). Since the majority of peptides present in the venom of *P. bundokalbo* have masses ~3-5 kDa, similar to the majority of tarantula-venom peptides adopting a highly stable inhibitor cystine

knot (ICK), enabling it to resist to chemical, protease, and thermal degradation, making them promising pharmacologic leads against voltage-gated ion channels [37,38].

There are three (3) known toxins identified from *Phlogius* sp., namely μ-theraphotoxin-Phlo1a, Phlo1b, and Phlo2a, all of which are gating modifier toxins of human voltage-gated sodium channels [39]. These toxins were extracted from Australian tarantula *Phlogius* sp., which has the same genus with Australian tarantula, *Phlogius crassipes* [39]. Interestingly, μ-theraphotoxin-Phlo1a, a spider toxin containing 35 amino acid residue with inhibitor cystine knot motif isolated from *Phlogius* sp., an Australian tarantula, has an average reduced mass of 4112.76 Da [41] as seen in Arachnoserver database, has similar mass as 4112.65 Da peptide (Supplementary Information) present in the LC-MS profile of the crude venom of *P. bundokalbo*, an endemic tarantula species in the Philippines. The small difference might be due to the sensitivity of the mass spectrometric instrument used. Based on its mass similarity with μ-theraphotoxin-Phlo1a, peptides in the venom fractions might also contain peptides with inhibitor cystine knot (ICK), a three-disulfide architecture of disulfide bonding pattern [37,38]. These spider venom peptides, ~3-5 kDa, had been seen in the 30-50% acetonitrile gradient in LC-MS, which is of similar HPLC gradient where venom fractions 2-5 were isolated. Based on the peptide masses generated, it can be deduced that its spider venom peptides might contain 27-40 amino acid residues, which are remarkably found in spider venom peptides tapped for potential drug candidates due to anticancer and antinociceptive properties [12]. For example, host defense peptides (~12-50 amino acids), containing a large portion of hydrophobic residues due to the presence of multiple arginine, lysine, and histidine [42]. These short amphiphilic spider peptides, such as latarcins, cupiennin 1a, and oxyopinins, work via a pore-forming mechanism and increases cancer-selective toxicity by forming several types of secondary structures that promote electrostatic interaction that enables these peptides to easily insert into the membrane phospholipid layer [12,14,43]. Given that spider venom peptides in the crude venom having distinct similar mass as μ-theraphotoxin-Phlo1a, this study serves as a baseline to further explore and understand this peptide and its mechanism and tap its potential in developing targeted breast chemotherapeutic leads.

Although several studies have supported that spider venom peptides have demonstrated cytotoxicity either through inhibiting cell growth, impairment of cell membrane, induction of apoptosis, and decreased cell proliferation [9,33,34], different species of spider exhibit different toxicities depending on tissue types. MCF-7 is a cell line derived from a human breast adenocarcinoma, which contains a deletion in exon 3 of the caspase 3 gene [6,44]. Modulation of various voltage-gated ion channels such as Na^+, K^+, Ca^{2+} or Cl^- and even the transient receptor potential (TRP) [12,33,34] respond to changes in membrane potential in excitable and cancer cells such as MCF-7 and plays a vital role in the regulation of the cell cycle and proliferation of cancer cells, by controlling ion transport within the cell. The cytotoxic nature of venom fractions against MCF-7 cells may suggest that partial inhibition of plasma membrane Ca^{2+} ATPase mediates the efflux of calcium, which play a critical role in cell cycle regulation in cancer cells such as MCF-7 [45-47]. It was also observed in ProTx-1, a tarantula peptide that selectively blocks Cav3.1 from *Thrixopelma pruriens*, which accounts for Ca^{+2} influx mediated cancer cell proliferation [48,49]. Both extracellular calcium and functional membrane calcium channels are necessary for cell progression from G1 to S to facilitate downstream signaling [48,50]. While only the venom's peptide components were analyzed in this study, the possible effect of other non-enzymatic components, such as polyamines, of the venom capable of inducing cytotoxicity should not be ruled out. For example, a hydroxyphenyl based compound, PA366, isolated from Australian funnel-web spider venom, is selectively toxic against MCF-7 breast cancer cells which target ionotropic and glutamate receptors [43,51].

Morphological changes such as cell detachment, cell rounding, cytoplasmic condensation, and cell shrinkage of MCF-7 cells upon exposure to venom fraction 3 (Figures 5A-C), are most likely the result of cell death, either through necrosis, apoptosis, or other programmed modes of cell death. Although venom peptides of *P. bundokalbo* have not been well-characterized in this study, previous reports on Philippine tarantula peptides had demonstrated cytotoxicity by inducing caspase 3/7 activation and pro-oxidative and nitrosative induction [27,52]. The synergistic effect of the interaction of venom components such as ions, low molecular weight compounds, enzymes, neurotoxins, and cationic peptides in the cell membrane surface may be due to high negative surface charge and higher transmembrane potential level of cancer cells compared to normal cells similar to the activity of spider toxins CsTx-1, cecropins and magainins [53-55]. The presence of negatively charged phosphatidylserine, phosphatidylethanolamine, sialic acid, or heparan sulfate in the surface of cancer cells might contribute to the preferential binding of spider venom peptides present in the venom fractions with MCF-7 cells [12,33,34].

The sPLA2 is a principal contributory to spider's prey capture and defense and other venomous animals such as snakes and scorpions. It plays a significant role in mediating

various cellular processes, including cell proliferation, differentiation, apoptosis, and inflammatory response [17]. Its role in triggering inflammatory conditions might be deduced from the sPLA2 generation of lysophosphatidylcholine-enriched high-density lipoprotein particles that modulate platelet function [56]. Moreso, sPLA2 is known to inhibit epidermal growth factor-induced receptor (EGFR) activation [17,19]. The growth of breast cancer cells such as MCF-7, is controlled by estrogen, progesterone, and plasma membrane-associated growth factor receptors, such as EGFR [6]. Remarkably, venom fraction 3 exhibits the highest cytotoxicity (IC_{50} 52.25 µg/ml) (Figure 4), which also showed a similar highest sPLA2 activity with venom fraction 5 (Figure 6). sPLA2 is activated by the presence of histidine/aspartic acid dyad in a Ca^{+2} dependent manner [57]. sPLA2 may also stimulate the release of excitatory amino acids (EAA) [17], which might result from excitatory neurotoxicity present in the venom fractions, although its exact mechanism of action remains unclear. However, its involvement may not be solely due to sPLA2 but the interaction of peptides and other venom components. The presence of histidine/aspartic acid had been found in several spider venom toxins exhibiting cytolytic activity such as ctenitoxins, sicaritoxins, lyocotoxins, miturgitoxin, oxotoxin, and zotatoxin [15,22,33,43]. The specific interaction of PLA2 present in spider venom components towards lipid membranes with tryptophan on the lipid-binding surface displays the highest activity toward neutral lipid substrates, whereas PLA2, with an excess of basic residues on the lipid-binding surface, interacts mainly with negatively charged surfaces as with cancer cells, such as MCF-7 [57]. The cytotoxic nature of venom fractions 3 and 5 may not solely be due to sPLA2 but with interactions to other components, low molecular weight compounds, enzymes, neurotoxins, and cationic peptides, which, when the specific bioactive compound has been purified, can be further explored to enhance its specificity and mechanism of reduced proliferation towards MCF-7 cells.

Spider venoms have been characterized to have exquisite potency and selectivity and are naturally rich sources of ion channel-acting toxins (neurotoxins) that inhibit presynaptic voltage-gated ion channels [61]. Neurotoxins from spider venom commonly induce a depressant response when synaptic transmission is inhibited [10,61,62]. Remarkably, the spider venom fractions from *P. bundokalbo* exhibited scores of 0.5-2.0 (Figure 7), indicating an excitatory response. The neurotoxins present in its venom fraction might be an activator of presynaptic voltage-gated ion channels, such as an agonist or gating modifier toxins that slows down channel inactivation similar to spider toxins [61-63]. This neuroexcitatory response can also be due to blockade of Ca^{+2}-activated K^{+} channel, positive allosteric modulator, competitive agonist, or antagonist of insect nicotinic acetylcholine receptors similar to other spider toxins [42,59,63-65]. Further purification and structure-function analysis should be done to clarify its direct mechanism of action with individual receptors mentioned.

Conclusion

There is clear evidence for the presence of venom peptide components of *P. bundokalbo* with cytotoxic, phospholipase A2, and neuroactive properties suggesting that its venom components can be characterized and elaborate on the structure-function to tap its potential in the development of targeted breast chemotherapeutic leads.

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MRSB conceived this research, designed and performed the experiments, analyzed and interpreted the data, and wrote the paper and participated in the revisions of it. GDTP collected and identified the spiders supervised by OMN, SML analyzed the LC-MS data, LAG extracted the venom, review and revise the manuscript. LAS is the research adviser of MRSB and helped in the review and revision of the manuscript. All authors read and approved the final manuscript.

Ethics approval

Wildlife Gratuitous Permit (R10 2017-35) was granted by the Deparment of Environment and Natural Resources (DENR) Region 10 for the collection of spiders.

Consent for publication

Not applicable.

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