

Article

Contact toxicity of various *Stemona collinsiae* root extracts against *Periplaneta americana* (Dictyoptera: Blattodea) and detection of didehydrostemofoline distribution in tissue using MALDI Imaging mass spectrometry

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Simple Summary: *S. collinsiae* extract showed insecticidal activity against pests and insect vectors such as *Spodoptera littoralis*, *Plutella xylostella* and *Parasarcophaga ruficornis*. But, it has never been tested in *Periplaneta americana*, an important insect vector, a transmitter of pathogenic and non-pathogenic microorganism and a good allergen. This research aimed at the detection of the insecticidal activity of *S. collinsiae* roots against *P. americana*, especially in final-instar nymph and adult stages which normally found in outdoor and indoor. The roots of *S. collinsiae* were extracted with sequentially reflux extraction method and several solvents as hexane, dichloromethane, ethanol and water. Hexane, dichloromethane, ethanol and water crude extracts were tested in the both stages of *P. americana* using topical application method. The dichloromethane extract presented the highest strength against the both stages of *P. americana*. Didehydrostemofoline, possessing acetylcholinesterase inhibitory activity, was found in dead *P. americana* contacting the solution of the dichloromethane extract after detecting with MALDI IMS. Didehydrostemofoline could penetrated through integument, widely distributed in *P. americana* tissue and bound with target site. Signs of toxicity and mortality were occurred. Thus, the dichloromethane extract could be an alternative active insecticide in spray and aerosol formulations. Didehydrostemofoline could be a chemical marker for quality control.

Abstract: Contact toxicity against *Periplaneta americana* has never been tested with *S. collinsiae* root extract. Hexane, dichloromethane, ethanol and water extracts were tested in final-instar nymphs and adult *P. americana* by topical application method. The dichloromethane extract showed the highest potency of contact toxicity against the final-instar nymphs (41-100% corrected mortality at 48 hours), lowest LC₅₀ (1.5±0.2 %w/v at 48 hours), and lowest LT₅₀ (36.1±0.8 hours at 10%w/v) while the water crude extract lacked the contact toxicity (0-0% corrected mortality at 48 hours). Signs of toxicity, such as excited movement, trembling body, motionlessness, and swollen abdomen segment including irregularly extended foregut were found at the both stages of *P. americana* dropping with solutions of dichloromethane extract. Detection of didehydrostemofoline distribution using IMS revealed that didehydrostemofoline distributed in the tissue of the dead final-instar nymph and adult *P. americana* contacting with dichloromethane extract, but it was not found in tissue of euthanized *P. americana* which exposed to the water extract. Didehydrostemofoline in the extract was a cause of toxicity signs and death of *P. americana* via a contact route. Thus, dichloromethane extract and didehydrostemofoline could be used as an active ingredient and chemical marker in aerosol and spray formulations for cockroach control.

Keywords: *Stemona collinsiae*; Non-Tai-Yak; Topical administration; Didehydrostemofoline; Alkaloids; Cockroach; Insecticide; MALDI IMS

1. Introduction

Periplaneta americana (American cockroach) is found in Thailand and many countries [1,2]. *P. americana* are an important host and carrier of several microorganisms [3-6]. *P. americana* receive many non-pathogenic and pathogenic microorganisms from unsanitary environments via ingestion or contact. The microorganisms live and propagate within internal organs such as the saliva gland and alimentary canal. Pathogenic microorganisms such as *Entamoeba histolytica* [7], *Aelurostrongylus abstrusus* [8], *Eimeria tenella* [9], and *Toxocara canis* [10] are transferred to humans and animals via mechanical and biological transmission. After humans or animals acquire the pathogenic microorganisms, insect vector-borne diseases occur [11]. Recently, SARS-CoV-2, a cause of coronavirus disease 2019 (COVID-19), has been found in patient stool [12], untreated wastewater and river water [13-15]. The quantity of virus which found can be used to monitor and predict COVID-19 prevalence in a community based on wastewater-based epidemiology [13, 16]. Sewage is a dwelling of cockroaches and it is possible that cockroaches can come into contact with the virus and act as a transmitter of COVID-19 [12, 17]. *P. americana* and their organs, including integument, egg, secretion, faeces, and the carcass are allergenic and can stimulate the immune system [18-21]. The allergen can result in a rash, itching, allergic rhinitis, and asthma in humans [20-22]. Cockroach phobia, or katsaridaphobia, is a fear of *P. americana* [23]. *P. americana* can affect the physical and mental of patients and lead to public health issues. The present research is interested in *P. americana*, a medically important insect vector, a good allergen, and a stimulant of phobia. Cockroach populations can be controlled through termination. Chemical insecticides, typically used, is necessary to eliminate large numbers of cockroaches. Cockroach control sprays containing insecticide are commonly found on the market and are easy to use. With these, small droplets of insecticide solution are sprayed and widely diffused in the air. When the droplets are exposed to the cockroach integument, the insecticide penetrates through the cuticle, enters the systemic circulation, distributes to the body tissues, interacts with the target site, and then signs of toxicity are evoked. The cockroaches are then killed. Chemical insecticides can be categorised as synthetic chemical insecticides and natural insecticides. Natural insecticides are called biopesticides [24]. Some natural insecticides have a modified chemical structure and mimic natural insecticidal activity such as neonicotinoids and pyrethroids. Agriculture is a large part of the Thai economy and several insecticidal plants have been used traditionally for a long time. *Stemona collinsiae* (Stemonaceae), an insecticidal plant, is used during plantation to prevent pepper vine damage due to pests [25]. Recently, crude extracts of *S. collinsiae* including didehydrostemofoline alkaloid have been found to exhibit pesticidal and insecticidal activities against *Spodoptera littoralis* [26], *Plutella xylostella* [27, 28], and *Parasarcophaga ruficornis* [29]. Nonetheless, the insecticidal activity of *S. collinsiae* crude extract against *P. americana* via contact administration has not yet been tested and reported. Final-instar nymphs and adult *P. americana* are mobile vectors and are usually found both indoors and outdoors. Thus, the nymphicidal and adulticidal activities of *S. collinsiae* root extracts against *P. americana* are investigated in this research. The aim of the present research is to detect the insecticidal activity of various *S. collinsiae* crude extracts such as hexane, dichloromethane, ethanol, and water crude extracts containing different phytochemicals against final-instar nymph and adult *P. americana* using a topical application method. Contact toxicity is important for the development of aerosol and spray insecticidal products. Insecticide chemicals attached to integument come to penetrate the tissue and are distributed to other organs. The chemicals interact at the target site and actions are shown. The distribution of didehydrostemofoline in *P. americana*'s tissue is detected using the matrix assisted laser desorption ionization-imaging mass spectroscopic

method (MALDI IMS). The IMS is high resolution technique to understand the distribution of a substance in different tissue regions and distribution mapping is produced. IMS involves a combination of techniques, including the thin tissue preparation technique, mass spectrometric technique, and microscopic technique including interpreting with software. MS spectra, molecular ion images with individual spots (pixel array) and single ion images of an interested compound are presented [30-32]. The MALDI IMS clearly presents the distribution of didydrostemofoline in tissue compared to the reference standard and other chemicals in chemistry databases. The efficacy of *S. collinsiae* root extracts against final-instar nymph and adult *P. americana* is evaluated and considered on the basis of percent of corrected mortality, median lethal concentration (LC₅₀), median lethal time (LT₅₀), and onset of action including signs of toxicity based on severity. This study presents the nymphicidal and adulticidal activities of *S. collinsiae* root extract, including the appearance of didydrostemofoline in tissue which is a novelty of natural insecticide against *P. americana* via contact administration.

2. Materials and Methods

2.1. Plant Materials [33]

Stemona collinsiae roots were harvested from Ubon Ratchathani, Thailand between December 2018 and January 2019. The herbarium of the roots and aerial parts were prepared for identification. The roots were cleaned with water and then dried using an electric fan to eliminate excess water on the surface of the roots. The dried roots were cut up into small pieces and were then dried in a hot air oven at $55 \pm 1^\circ\text{C}$ for 72 hours. The small dry pieces of root were ground and sieved using a #30 mesh sieve. The powdered root was used in further reflux extraction processes.

The herbarium of *S. collinsiae* roots and aerial parts were identified and deposited in the Forest and Plant Conservation research office, Department of National Parks, Wildlife and Plant Conservation with voucher specimens BKF No. 196976.

2.2. Chemicals and reagents

Hexane, dichloromethane, ethanol, and acetone in AR grade were purchased from RCI Labscan Limited, Bangkok, Thailand. Water type I was used in the experiment and was produced by Milli-Q®, Germany. All reagents were analytical grade.

2.3. Preparation of hexane, dichloromethane, ethanol, and water crude extracts using the sequentially reflux extraction method modified from [29]

Hexane, dichloromethane, ethanol, and water crude extracts used in the previous experiment [33] were used again in this experiment. The detection of phytochemicals in each extract was tested with the thin-layer chromatography method, with the method and results described in [33]. A brief explanation of the extraction method follows. Hexane, dichloromethane, ethanol, and purified water were used as extractants. Hexane was used as the first extractant. The powdered root (300 g), contained in a round-bottom flask, was soaked in the hexane (1000 ml). The top of the flask was covered with a cold condenser. The flask was soaked in a warm water bath controlling temperature at $60\text{--}70^\circ\text{C}$ for 1 hour. The liquid extract was filtered with a filter paper. The liquid filtrate was collected in a bottle protected from light. The filtrate (2 ml) was pipetted and tested for alkaloids using thin-layer chromatography (TLC) and Dragendorff's spray reagent. A new hexane was added into the residue again. The flask was re-immersed in the water bath for 1 hour. The hexane extraction and filtration process were repeated until all alkaloids were exhaustively extracted from the residue. The complete hexane extraction was confirmed by TLC and Dragendorff's spray reagent. The collected filtrates were concentrated using a rotary evaporator under reduced pressure at $40 \pm 1^\circ\text{C}$. Then, the hexane crude extract was dried on a water bath at $70 \pm 1^\circ\text{C}$, poured into a tightly sealed glass container, protected from light, and kept in a refrigerator at 4°C for the next experiment. After the hexane extraction was completed, the hexane was changed to dichloromethane. For dichloromethane, ethanol, and water extraction, the extraction process was

repeated using the same steps as described in the hexane extraction. The water crude extract was dried using lyophilization.

2.4. Ethical consideration statement

The study was approved by Ethics Committee and accordance with ethical principles and guidelines for the use of animals provided by the National Research Council of Thailand. All experiments were performed in accordance with protocols approved by the Animal care and Use Committee of Thammasat University: Protocol No. 005/2020 and the Animal care and Use Committee of Faculty of Tropical Medicine, Mahidol University: Protocol No. 001/2021 Certificate No. FTM-ACUC 005/2021.

2.5 *Periplaneta americana* rearing [33]

Periplaneta americana was collected from Ratchaburi, Thailand. The cockroach species was identified by Yudthana Samung, an entomologist who works at the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University. Species identification was performed following the handbook of domiciliary cockroach species in Thailand [34]. *P. americana* were reared by Anon Phayakkaphon who works in the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University. The cockroaches were fed water and dry cat food (Purina® Friskies® for adult cats, Nestlé, Thailand). They were maintained and bred in plastic boxes (30 × 30 × 30 cm) with lids on the top. Petroleum jelly was smeared on the inside wall of each plastic box to prevent the cockroaches from escaping. The plastic boxes containing *P. americana* were placed in a cockroach-rearing room at the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, under ambient temperatures (27–30°C), 70%–90% humidity, and a photoperiod of a 12:12 hour dark: light cycle. Final-instar nymphs and adult *P. americana* were used for contact toxicity tests with the topical application method.

2.6. Contact toxicity using the topical application method modified from [35, 36]

2.6.1. Sample preparations

Each extract was diluted and a series of dilution was prepared. A range of concentration from 1.0–12.0% w/v (g/mL) was tested. Two-fold serial dilutions were prepared (six concentrations per two-fold dilution). The range of concentration of the extract solution, producing a range of corrected mortality between 10–90% (Finney, 1971), was selected to calculate LC₅₀. The hexane and dichloromethane crude extracts were dissolved in acetone while the ethanol and the water crude extracts were dissolved in 70% ethanol. All *P. americana* in the negative control groups received drops of only acetone instead of the extract solutions.

2.6.2. Bioassay modified from [35]

Final-instar nymphs (10 nymphs, unsexed nymphs) and adult cockroaches (10 cockroaches, mixed sexes) were used for this experiment. Before dropping the extract solution or the acetone on the first segment of abdominal sternites, the nymphs and adult *P. americana* were anaesthetised and chilled in a refrigerator at -20°C for 10–15 minutes and the cockroaches were frequently checked to avoid death. Chilling was selected to avoid chemical exposure. Each concentration of the solution, in volumes of 20 µL, was dropped on the first abdominal segment of each cockroach. The same technique was used for the negative control group. All the *P. americana* groups were kept under the same conditions. Mortality of final-instar nymphs and adults was observed at 1, 2, 6, 12, 24, 48, 72, 96, and 120 hours after treatment. Dead *P. americana* were detected by prodding them with forceps. Signs of death were as follows; *P. americana* did not respond to prodding, were immobile, and were unable to return to a normal posture. All experiments were performed with three replications.

2.7. Detection of median lethal Time (LT₅₀) at specific concentration

Three specific concentrations as the lower values than the minimum concentration (0.01% w/v), LC₅₀ (1.5% w/v) which possessed the highest percentage of corrected mortality and the lowest LC₅₀ concentration in the final-instar nymph, and the lower values

than the maximum concentration (10%w/v) were tested in both stages of *P. americana*. The hexane and dichloromethane extracts were dissolved in acetone while the ethanol and water extracts were dissolved in 70% ethanol. Vortex mixer was used for dissolving the extract. A clear solution was produced. The experiment was performed in the final-instar nymphs (10 nymphs, unsexed nymphs) and adult cockroaches (10 cockroaches, mixed sexes) with the same method described in Bioassay. Mortality and signs of toxicity were detected at 1, 2, 4, 6, 24, 48, 72, 96, and 120 hours. The results of each extract were compared. LT_{50} was calculated using Probit analysis as described in Parameter and statistical analysis was performed. All experiments were performed in three replications.

2.8. Detection of the dissected alimentary canals in the dead *P. americana* using dissection method in the extract-treated group and negative control group

Dead *P. americana* were dissected and their alimentary canals removed. *P. americana* in the negative control group were killed by freezing in a refrigerator at -20°C before dissection, then fixed onto the dissection board with pins. Dissection was performed by longitudinally cutting from the abdomen using medical scissors and the exoskeleton and fat tissue were gradually removed using forceps. The alimentary canals from *P. americana* in the extract-treated group were compared with those from the negative control group. The images of *P. americana* and the dissected alimentary canal were taken using Canon EOS 500D digital SLR camera (Canon, Japan). SNZ745T stereomicroscope 10x (Nikon, China) with microscope camera MDX503 and iWorks software (Lanoptik Technologies Ltd., China) was used for image enlargement.

2.9. Parameters and statistical analysis

Percentage of observed mortality was calculated. The data was corrected using Abbott's formula [37]. The percentages of corrected mortality were calculated and shown as mean \pm SD or range as min-max. Signs of toxicity, morphological changes, abnormal behaviour, and onset of action were observed and recorded. Time-to-event was calculated using survival analysis (IBM SPSS statistics version 28.0.0, IBM Corporation, USA). The morphological and behaviour changes including sign of toxicity were considered and divided into groups. The percentage of the *P. americana* in each group was calculated presented in the range of min and max or mean \pm SD. Probit analysis using Probit analysis program, March 1987 version [38] and IBM SPSS statistics version 28.0.0 (IBM Corporation, USA) were used to calculate median lethal Time (LT_{50}). LC_{50} and LT_{50} were calculated at 95% confidence limit of upper (UCL) and lower (LCL) confidence limits. Results with p-value <0.05 are considered statistically significant.

2.10. Distribution of didehydrostemofoline in the tissue of the cockroaches using the MALDI imaging mass spectrometry (IMS) technique

2.10.1 Preparation of didehydrostemofoline reference substance

Didehydrostemofoline was dissolved in dichloromethane and adjusted to a final concentration of 0.2 mg/mL. The solution was detected with MALDI IMS qualitative analysis. The MS spectra, molecular mass and fragment pattern of didehydrostemofoline reference substance were produced and this information was used to search for didehydrostemofoline in the *P. americana* tissue.

2.10.2 Preparation of the tissue and cryosectioning

The highest percent of corrected mortality obviously was shown in the group of *P. americana* that received the solution of dichloromethane crude extract, whereas all of the *P. americana* treated with the water crude extract were not killed by the water crude extract. Thus, in the detection of didehydrostemofoline in the tissue using IMS, the *P. americana* in the dichloromethane extract-treated group were detected and compared with the surviving *P. americana* in the water extract treated-groups. After dropping the dichloromethane extract solution, *P. americana* which had clearly the symptom of swelling abdomen were detected for this test. The surviving *P. americana* in contact with the water crude extract were euthanised by freezing in a refrigerator at -20°C for 1-2 hours before

sectioning. Dead final-instar nymphs and *P. americana* were transported to National Science and Technology Development Agency (NCTC) for the detection of didehydrostemofoline distribution using MALDI IMS. The *P. americana* were firstly separated to three sections of the head, thorax, and abdomen because the size of *P. americana* was bigger than slide. Each part was embedded in optimal cutting temperature (OCT) compound (FSC 22 Blue Frozen Section Compound, Leica, USA) and sectioned at 50 μm using cryostat microtome (CM1950, Leica, Germany). The sectioned tissue was prepared on an indium thin oxide coated slide glass (ITO slide) (Sigma Aldrich, St. Louis, United States).

2.10.3 Coating section with matrix

The sectioned tissue on ITO slide was automatically sprayed with α -Cyano-4-hydroxycinnamic acid (CHCA) (Sigma Aldrich, Darmstadt, Germany) using iMLayer (Shimadzu, Japan). The film thickness was controlled at 0.7 μm , deposition time was set at 8 minutes, and deposition temperature was controlled at 250°C.

2.10.4 MALDI IMS Analysis

Analysis was performed using imaging mass microscope (IMS) (iMScope TRIO, Shimadzu, Japan) with laser-diode-excited Nd (YAG laser). MALDI-ESI Ionization method was used in the experiment. For MS acquisition parameters, positive mode of ion polarity was selected. Pitch was set at 37 μm . Mass of substances was detected in range of 382–390 m/z . Sample voltage and detector voltage were set at 3.00 and 2.00 kV, respectively. For laser firing parameters, the number of shots was set to 100 shots and the repetition rate was controlled at 1000 Hz. Diameter and intensity of the laser was fixed at 2 and 20.0, respectively.

2.10.5 Image processing and data analysis

Imaging MS solution version 1.30 software (Shimadzu, Japan) was used for image processing. Data analysis was performed by ACD Labs 2018 to search for a mass (m/z) chemical structure within the PubChem and ChemSpider databases (> 90 million structures). The MS spectra, molecular mass, and fragment pattern of didehydrostemofoline distribution in the tissue were compared with didehydrostemofoline reference substance.

3. Results

3.1 Contact toxicity using topical application method

For the tested concentration range of 1-12% w/v, the highest corrected mortality percentage was found in the group of final-instar nymphs treated with dichloromethane extract (41.0-100.0% corrected mortality at 48 hours), followed by the hexane extract-treated group (43.0-83.0% corrected mortality at 48 hours). The corrected mortality percentage values of adult *P. americana* in the dichloromethane and hexane extract-treated groups were 23.0-46.0% and 17.0-43.0% respectively. Dichloromethane crude extract possessed the lowest LC_{50} against final-instar nymphs ($1.5 \pm 0.2\%$ w/v at 48 hours), followed by hexane crude extract ($2.1 \pm 0.3\%$ w/v at 48 hours). Contrary to the adult *P. americana*, the LC_{50} could not be calculated at the same range of tested concentrations. The ethanol extract slightly killed final-instar nymphs (0.0-10.0% w/v at 48 hours). The water crude extract could not kill both stages of all *P. americana* ($0.0 \pm 0.0\%$ corrected mortality), similar to *P. americana* in the negative control group ($0.0 \pm 0.0\%$ corrected mortality).

3.2 Detection of median lethal Time (LT_{50}) at specific concentrations (0.01, 1.5, and 10.0% w/v)

At lower values than the minimum tested concentration (0.01% w/v), final-instar nymphs and adult *P. americana* in all groups survived and LT_{50} could not be calculated. However, at this concentration, 0.05% of the adult *P. americana* that contacted only the dichloromethane extract showed signs of toxicity, with their bodies rising up, hind legs frequently scratching the abdomen and body shaking. After a while, these signs dissipated and the insects survived.

At concentration of LC₅₀ (1.5% w/v), the LT₅₀ value was presented in the group of final-instar nymphs (47.3±6.8 hours) and adult *P. americana* (97.5±8.5 hours) exposed to the dichloromethane extract, while the hexane, ethanol and water extracts did not kill both stages of all *P. americana*. Onset of the excited movement and body shaking, onset of immobility and onset of swollen abdomen in adult *P. americana* receiving the dichloromethane extract were at 0.4±0.02 hours (0.39-0.48 hours), 29.4±8.7 hours (12-46 hours) and 27.8±0.6 hours (27-29 hours), respectively.

At lower values than the maximum tested concentration (10% w/v), the dichloromethane extract greatly affected final-instar nymphs and produced the lowest LT₅₀ values (36.1±0.8 hours), while the ethanol and water extracts did not affect the both stages of *P. americana* and LT₅₀ could not be calculated, similar to the negative control exposed to acetone only. Hexane and dichloromethane extracts at concentration of 10% w/v were able to kill adult *P. americana* at LT₅₀ of 79.7±19.5 and 62.2±2.9 hours, respectively. At the same concentration, LT₅₀ values in the final-instar nymph groups were shorter than for adult *P. americana*. In the group of final-instar nymphs and adult *P. americana* receiving dichloromethane extract at concentration of 10% w/v, onset of excited movement and body shaking occurred at 0.5±0.01 hours (0.52-0.53 hours) and 0.5±0.03 hours (0.40-0.51 hours), respectively. Onset of immobility was at 1.6±0.1 hours (1.5-1.7 hours) and 1.7±0.2 hours (1.4-2.0 hours) for the final-instar nymph and adult *P. americana*, respectively. Onset of obvious swollen-abdomen symptom was at 1.6±0.1 hours (1-2 hours) and 15.5±7.1 hours (22-29 hours) for the final-instar nymph and adult *P. americana*, respectively. The results are shown in Table 1.

Table 1. The LC₅₀, LT₅₀, and onset of actions of the crude extracts affecting to the both stages of *P. americana*

	Concentration (%w/v)	Final-instar nymph					Adult <i>P. americana</i>				
		Hexane	Dichloromethane	Ethanol	Water	Negative control	Hexane	Dichloromethane	Ethanol	Water	Negative control
% corrected mortality at 48 hours ¹	1-12	43.0-83.0	41.0-100.0	0.0-10.0	0.0-0.0	0.0-0.0	17.0-43.0	23.0-46.0	0.0-0.0	0.0-0.0	0.0-0.0
LC ₅₀ at 48 hours (%w/v) ²	1-12	2.1±0.3	1.5±0.2	ND	ND	ND	ND	ND	ND	ND	ND
LT ₅₀ (Hours) ²	0.01	ND	ND	ND	ND	ND	ND	ND*	ND	ND	ND
	1.5	ND	47.3±6.8	ND	ND	ND	ND	97.5±8.5	ND	ND	ND
	10	48.0±9.2	36.1±0.8	ND	ND	ND	79.7±19.5	62.2±2.9	ND	ND	ND
Onset of excited state (Hours) ²	1.5	ND	ND	ND	ND	ND	ND	0.4±0.02	ND	ND	ND
	10	ND	0.5±0.01	ND	ND	ND	3.3±0.0	0.5±0.03	ND	ND	ND
Onset of immobility (Hours) ²	1.5	ND	15.1±4.2	ND	ND	ND	43.3±8.6	29.4±8.7	ND	ND	ND
	10	2.0±1.0	1.6±0.1	ND	ND	ND	6.0±0.0	1.7±0.2	ND	ND	ND
Onset of clear swollen abdomen (Hours) ²	1.5	ND	24.0±0.0	ND	ND	ND	48.5±0.0	27.8±0.6	ND	ND	ND
	10	3.0±1.0	1.6±0.1	ND	ND	ND	23.9±0.2	15.5±7.1	ND	ND	ND

¹ presented as range of min and max

² presented as average ± SD

ND* = not detectable but the signs of toxicity were shown as body rising up, scratch abdomen part and body shaking

ND = not detectable

Final-instar nymphs and adult *P. americana* in the ethanol extract-treated group showed predominant apathy and slow movement symptoms, compared with adult *P. americana* in the negative control group that exhibited fast movement and good response to stimuli. The ethanol crude extract slightly affected adult *P. americana* when compared with final-instar nymphs. After treatment of the ethanol extract, final-instar nymphs and adult *P. americana* presented apathy and motionless symptoms that later dissipated and the insects did not die. After 48 hours, *P. americana* in the ethanol extract-treated group survived. Final-instar nymphs and adult *P. americana* receiving water extract at all concentrations

did not show any signs of toxicity and all *P. americana* survived. All the final-instar nymphs and adult *P. americana* in the negative control group exposed to acetone only showed no signs of toxicity and they survived.

After *P. americana* received the dichloromethane extract, excited movement with a motionless and swollen abdomen occurred. The swollen-abdomen symptom was irreversible and found in all dead *P. americana* in both stages (Figures 1).

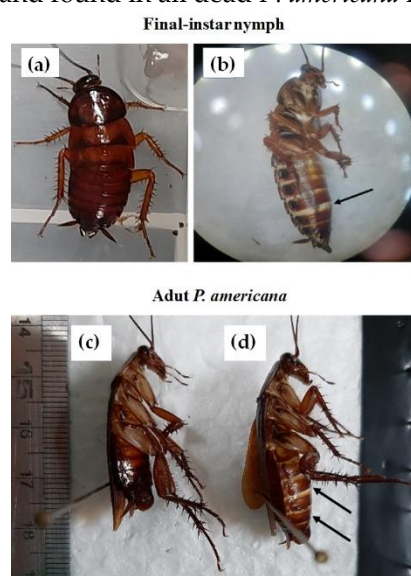


Figure 1. the swelling of the abdomen segment in the both stages:

(a) Final-instar nymphs in the negative control group dropping with acetone only, swelling of the abdomen segment did not occur.

(b) Final-instar nymphs in dichloromethane extract-treated group produced visible swelling of the abdomen segment, with extension of the intersegmental membrane (black arrow)

(c) Adult *P. americana* in the negative control group dropping with acetone only, swelling of the abdomen segment did not occur.

(d) Adult *P. americana* in the dichloromethane extract-treated group showed swelling of the abdomen segment, with extension of the intersegmental membrane (black arrow).

After the final-instar nymphs and adult *P. americana* received the extracts, the occurrence of signs of toxicity was divided into three categories based on the severity of the symptoms as category I: normal defence effect consisting of high sensitivity, good response to stimuli and fast movement, category II: depression, apathetic and motionless and category III: swollen abdomen, indicating the highest severity and mortality in all *P. americana* receiving dichloromethane crude extract and hexane crude extract because the swelling was irreversible. The classification and percentage of each category are shown in Table 2.

At 24 hours, final-instar nymphs, dropped with hexane and dichloromethane extracts, showed the defence effect (16.7-40.0 and 8.3-56.7%), depression with immobility at (0.0-10.0 and 0.0-26.7%) and swollen abdomen (16.7-43.3 and 20.0-100.0%). Adult *P. americana* presented in the same manner (Table 2).

At 48 hours, the percentage of defence effect with fast movement in the group of final-instar nymphs receiving the hexane and dichloromethane extracts (0.0-10.0% and 0.0-0.0%) reduced while the percentage of swollen-abdomen symptom increased (0.0-90.0% and 43.3-100.0%).

At 24-48 hours, signs of toxicity were not found in both stages of all *P. americana* receiving the water extract (0.0-0.0%) and all *P. americana* in the negative control receiving only acetone (0.0-0.0%). All *P. americana* showed the greatest response to stimuli and fastest movement (100% of defence effect with fast movement), compared with *P. americana* in the hexane and dichloromethane extract-treated groups.

Table 2. Percentage of the occurrence sign of toxicity expressing in each group of *P. americana*, observed at 24th and 48th hour

Group of the cockroaches	Time of observation	The percent of the signs of toxicity, observed at 24 th and 48 th hour ¹					
		Final-instar nymph			Adult <i>P. americana</i>		
		Obviously Swollen abdomen	Apathetic and motionless	Defence effect with fast movement	Obviously swollen abdomen	Apathetic and motionless	Defence effect with fast movement
Hexane extract-treated group	At 24 hours	16.7-43.3	0.0-10.0	16.7-40.0	0.0-90.0	5.0-80.0	0.0-10.0
		(35.0±10.1)	(3.3±0.4)	(30.7±9.2)	(51.0±3.9)	(48.0±2.5)	(5.5±0.4)
	At 48 hours	0.0-90.0	5.0-80.0	0.0-10.0	20.0-76.7	13.3-60.0	0.0-3.3
		(51.0±2.5)	(48.0±2.5)	(5.5±1.4)	(48.3±2.7)	(39.3±1.4)	(1.5±0.7)
Dichloromethane extract-treated group	At 24 hours	20.0-100.0	0.0-26.7	8.3-56.7	0.0-94.0	15.0-95.0	0.0-5.0
		(74.7±5.1)	(12.7±5.1)	(27.0±8.0)	(55.9±6.6)	(66.7±2.0)	(2.2±1.0)
	At 48 hours	43.3-100.0	0.0-80.0	0.0-0.0	0.0-85.0	26.7-83.9	0.0-0.0
		(78.0±3.4)	(33.9±2.0)	(0.0±0.0)	(43.5±37.8)	(63.1±2.4)	(0.0±0.0)
Ethanol extract-treated group	At 24 hours	6.7-10.0	22.2-90.0	0.0-11.1	0.0-0.0	0.0-80.0	0.0-60.0
		(7.3±2.2)	(60.4±2.6)	(5.0±2.2)	(0.0±0.0)	(32.0±2.1)	(32.9±2.4)
	At 48 hours	6.7-10.0	22.2-90.0	0.0-11.1	0.0-0.0	0.0-0.0	100
		(7.3±2.2)	(60.4±2.6)	(5.0±2.2)	(0.0±0.0)	(0.0±0.0)	(100.0±0.0)
Water extract-treated group	At 24 hours	0.0-0.0	0.0-0.0	100	0.0±0.0	0.0±0.0	100
		(0.0±0.0)	(0.0±0.0)	(100.0±0.0)	(0.0±0.0)	(0.0±0.0)	(100.0±0.0)
	At 48 hours	0.0-0.0	0.0-0.0	100	0.0±0.0	0.0±0.0	100
		(0.0±0.0)	(0.0±0.0)	(100.0±0.0)	(0.0±0.0)	(0.0±0.0)	(100.0±0.0)
Negative control group (Acetone only)	At 24 hours	0.0-0.0	0.0-0.0	100	0.0-0.0	0.0-0.0	100
		(0.0±0.0)	(0.0±0.0)	(100.0±0.0)	(0.0±0.0)	(0.0±0.0)	(100.0±0.0)
	At 48 hours	0.0-0.0	0.0-0.0	100	0.0±0.0	0.0±0.0	100
		(0.0±0.0)	(0.0±0.0)	(100.0±0.0)	(0.0±0.0)	(0.0±0.0)	(100.0±0.0)

¹ presented as mean of % at minimum concentration – mean of % at maximum concentration
(...) presented as mean±SD

3.3 Detection of the alimentary canals in the dead *P. americana* in each extract-treated groups comparing with the negative control group using dissection method

Dead final-instar nymphs and adult *P. americana* dropped with dichloromethane extract showed predominant physical symptoms as swelling of the abdomen (Figure 2a), while *P. americana* treated with the water extract (Figure 2b) and the negative control group did not show swelling symptoms (Figure 2c). After dissection of the both *P. americana* receiving the dichloromethane extract, their alimentary cavities at the foregut were swollen like a bubble and had a black residue in the foregut (Figure 2a). The swelling symptom and alimentary canal were also found in all *P. americana* receiving the hexane extract, similar to *P. americana* in the dichloromethane extract treated-group but figure did not show.

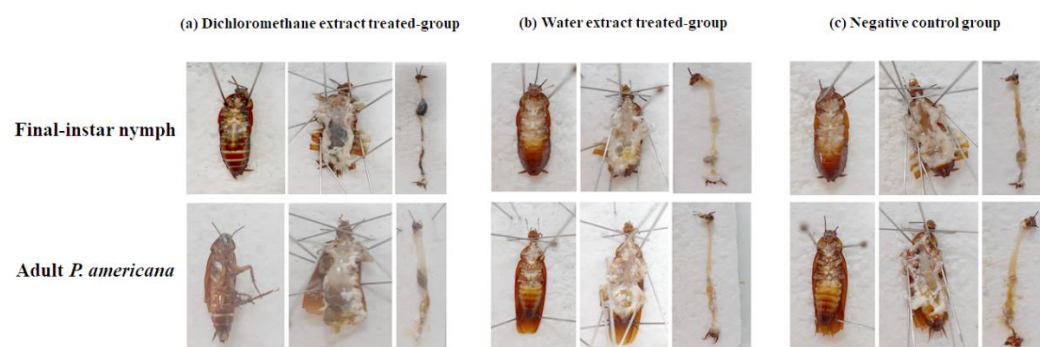


Figure 2. the swollen abdomen part, the dissected *P. americana* and the dissected alimentary canals from final-instar nymph and adult *P. americana* in (a) the dichloromethane treated-group, (b) the water treated-group and (c) the negative control group receiving acetone only

3.4 Distribution of didehydrostemofoline in tissue of the dead *P. americana* using MALDI imaging mass spectrometry (IMS) technique

P. americana which had the clear swelling symptom at abdomen were euthanized using freezing at -20 °C for 2 hours. Dead final-instar nymphs and *P. americana* were transported to NCTC. The didehydrostemofoline alkaloid reference substance ([M+H]⁺ 386.200), which was detected previously, was detectable and represented as blue and green spots in the IMS image (Figure 3). The distribution of didehydrostemofoline widely appeared in the IMS images of both stages of *P. americana* after treatment with the solution of dichloromethane crude extract (Figure 3a and 3c) but it was not found in the tissue of both stages of dead *P. americana* dropped with the solution of water extract (Figure 3b and 3d). The high intensity of the blue and green spots was found in the tissue of adult *P. americana* contacting the dichloromethane extract (Figure 3c) more than in the tissue of dead final-instar nymphs (Figure 3a). The highest intensity of didehydrostemofoline appeared in metathorax and followed by head segment.

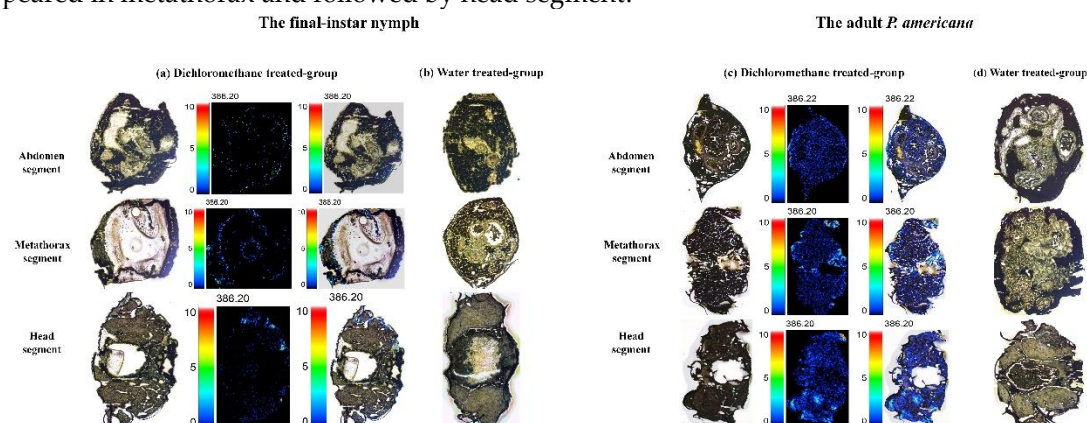


Figure 3. the distribution of didehydrostemofoline in the tissue:

- (a) Presence of didehydrostemofoline (blue and green spots) in final-instar nymphs exposed to dichloromethane extract solution.
- (b) Absence of didehydrostemofoline in final-instar nymphs exposed to the water extract solution.
- (c) Presence of didehydrostemofoline (blue and green spots) in adult *P. americana* exposed to the dichloromethane extract solution.
- (d) Absence of didehydrostemofoline in adult *P. americana* exposed to the water extract solution.

4. Discussion

Stemona collinsiae is a generally known insecticidal plant [26, 28]. Didehydrostemofoline, isolated from methanolic *S. collinsiae* leaf and root extracts, showed contact toxicity in the larvae of *Spodoptera littoralis* [26]. Pupation of *Parasarcophaga ruficornis* larvae was accelerated after dropping a solution of 70% ethanolic *S. collinsiae* root extract on their bodies. A segmental-like shape occurred and the adult flies could not hatch from the irregular pupae [29]. These scientific reports showed that *S. collinsiae* possessed contact toxicity that could eliminate pest and insect vectors. *P. americana*, an important medical insect vector that is resistant to insecticides and hard to kill [39-43] has never been tested with *S. collinsiae* root extracts. Nymphicidal and adulticidal activities of *S. collinsiae* against *P. americana* via topical administration are unknown. The insecticide activities of various *S. collinsiae* extracts against final-instar nymphs and adult *P. americana* via contact route, including distribution of didehydrostemofoline in their tissue, were new and basic knowledge. This novelty may lead to the development of active ingredients in spray and aerosol formulations in the future. The formulations supported the elimination of *P. americana* that preferred to live by escaping and crawling into crevices and narrow confined spaces [44].

In our previous research [33], *S. collinsiae* roots were extracted with different extractants using a sequential reflux extraction method based on arrangement of solvent polarity from low to high. Hexane, dichloromethane, ethanol and water crude extracts consisted of different phytochemical constituents and amounts of phytochemicals. Hexane and dichloromethane crude extracts contained didehydrostemofoline and unknown fluorescent substances. But, they were not present in the water crude extract. The highest content of these substances was found in the dichloromethane crude extract, followed by the hexane crude extract and slightly found in the ethanol extract. Hexane and dichloromethane crude extracts showed high potency of nymphicidal and adulticidal activities via oral administration. The ethanol crude extract slightly killed *P. americana* due to the effect of remained didehydrostemofoline in the extract. The water crude extract did not kill either stage of *P. americana*. The four extracts used in our previous research were tested again in this contact toxicity test. The dichloromethane extract possessed the highest strength of nymphicidal and adulticidal activities via contact administration and the shortest onset time, followed by the hexane crude extract, while all *P. americana* in the groups contacting water crude extract and the negative control applied with acetone only survived and did not show any signs of toxicity.

Signs of toxicity as excited movement, trembling and motionlessness including swollen abdomen were expressed in both stages of *P. americana* contacting the hexane and dichloromethane extracts. These signs of toxicity were also recorded in the group of *P. americana* eating bait containing the hexane and dichloromethane extracts. The swollen abdomen was an irreversible symptom and sign of death. The apathy and motionlessness symptoms occurred in the group of *P. americana* contacting the ethanol extract, similar to the group of *P. americana* ingesting bait containing the ethanol extract. The symptoms were a feature in all *P. americana* receiving the ethanol extract. *P. americana* receiving the water extract via oral and contact administrations did not show any signs of toxicity. The same signs of toxicity, expressed in both groups of *P. americana* ingesting and contacting the hexane and dichloromethane extracts, occurred because the phytochemicals had similar pharmacophoric patterns and interacted with the same type of biological target sites [45]. Didehydrostemofoline alkaloid, abundantly found in *S. collinsiae* [46-48], is a *Stemona* alkaloid that possesses acetylcholinesterase inhibitory activity [49-52] and was found in the dissected alimentary canal in our previous research. Recently, it was also found in tissue of both stages of *P. americana* contacting the dichloromethane extract after detection by the IMS method but not found in the tissue of euthanised *P. americana* exposed to the water extract. This showed that didehydrostemofoline alkaloid in the dichloromethane extract

possessed nymphicidal and adulticidal activities via contact administration. Didehydrostemofoline adhered on the insect integument and penetrated from the dropping area at the first abdomen segment, passed the epicuticle and other layers and distributed to other parts of the insect body. According to the Rule of 5 [53-57], didehydrostemofoline is a small molecule, having molecular mass (MW 385.5) less than 500 D and has lipophilic property of didehydrostemofoline (computed log P 2.4 less than 5, H-bond donors = 0 less than 5 and H-bond acceptors = 6 less than 10) [58]. Compounds, possessing molecular mass of around 324 and log P value ranging -0.5 to 3 can penetrate through membrane and accumulate in lipid membrane [56]. The rotatable bond of didehydrostemofoline (rotatable bond = 3) of less than 12, indicated that it was a flexible compound and promoted binding between the compound and target site [56]. Compared with neonicotinoids and other insecticides, nicotine (MW 162.2, computed Log P 1.2, H-bond donors 0, H-bond acceptors 2 and rotatable bond 1) [56, 59] is a natural insecticidal alkaloid, having a smaller molecule than didehydrostemofoline. It can be rapidly absorbed and penetrated through *P. americana* integument [60]. Imidacloprid (MW 255.7, computed Log P = 1.84, H-bond donors = 1, H-bond acceptors = 7 and rotatable bond = 3) [56] is poorly ionised in neutral media, can easily penetrate the cuticle of *Apis mellifera* [61]. Other neonicotinoids as dinotefuran (MW 203.22, computed Log P = 0, H-bond donors = 3, H-bond acceptors = 4 and rotatable bond = 5) [62] and acetamiprid (MW 222.7, computed Log P = 0.56, H-bond donors = 0, H-bond acceptors = 4 and rotatable bond = 4) [56, 63] can also quickly penetrate the integument of honey bees after contact [64]. Other insecticides such as malathion (MW 330.4, computed log P = 0.9, H-bond donors = 0, H-bond acceptors = 6 and rotatable bond = 11) [56, 65] penetrate through the cuticle, distributed to haemolymph and other internal organs after topical application on *Musca domestica* [66]. The IMS results suggested that didehydrostemofoline was widely distributed and accumulated in *P. americana* tissue. Didehydrostemofoline, lipophilic compound, dissolved in cuticular waxes [67] and moved across the lipophilic barrier (cement and wax layers) via the pore canals (average diameter = 0.15 μ) and dermal ducts [68], penetrated through other layers and distributed to haemolymph and other tissue [53-57]. The pyrethroids, tetramethrin (MW 331.4, computed log P = 3.43, H-bond donors = 0, H-bond acceptors = 5 and rotatable bond = 5) [56, 69] and resmethrin (MW 338.5, computed log P = 3.91, H-bond donors = 0, H-bond acceptors = 3 and rotatable bond = 7) [56, 70] enter the body through mesothoracic spiracles and the mesothoracic trachea [71]. Didehydrostemofoline, having MW 385.5, possibly entered the body of insects via spiracles. Mapping of the penetration of didehydrostemofoline using the IMS method should be performed in future experiments. However, the *S. collinsiae* root extract contains several alkaloids and other phytochemicals such as stemofoline, 2'-hydroxystemofoline, isostenine, neotuberostemonine and bisdehydroneotuberostemonine [72], rotenoids [73] and stilbenoids [74, 75]. Hydroxystemofoline and stemofoline also presented acetylcholinesterase inhibitory activity [49, 50]. The phytochemicals synergised a neurotoxic effect against *P. americana*. Didehydrostemofoline and other alkaloids having the same pharmacophore could bind to the same biological target sites. The same mechanism of action and signs of toxicity occurred in *P. americana* exposed to dichloromethane extract solution, similar to *P. americana* ingesting the extract. The mechanism of action of didehydrostemofoline alkaloid and other alkaloids in *P. americana* requires further study. An inflated peritoneum and serious tearing of the exoskeleton were not found in *P. americana* dropped with the solutions. The content of the used extracts and the amount of the active phytochemicals in the solution of hexane and dichloromethane extracts (1-12% w/v) were less than in the bait (1-50% w/w). But, it possible to happened when the concentration of the extracts was increased. From the results, concentration of the used crude extract and didehydrostemofoline was an importance.

Surprisingly, at concentration 0.01% w/v, signs of toxicity as tremor symptoms appeared in adult *P. americana* in the dichloromethane extract-treated group only, with no symptoms evident in the other groups. Some cockroaches recovered and this concentration did not kill all *P. americana*. Results confirmed that the dichloromethane crude extract possessed the highest adulticidal activity. The LC₅₀ values and LT₅₀ of the final-instar

nymphs were less than adult *P. americana*, indicating greater susceptibility of final-instar nymphs. When the concentration of the extracts was increased to LC₅₀ and maximum concentration, the mortality of *P. americana* correspondingly increased. At the maximum concentration, *P. americana* died with short period of onset, duration of action and time-to-death. Nymphicidal and adulticidal activities of the hexane and dichloromethane extracts via contact route directly related to the amounts of extract, didehydrostemofoline and unknown fluorescent substances. Concentration of the extract and didehydrostemofoline was an important because the concentration of the extract and didehydrostemofoline, less than effective concentration, could not kill *P. americana*, e.g., *P. americana* in the ethanol extract-treated group and the dichloromethane crude extract at concentration of 0.01%w/v. Higher concentrations of the extract solution accelerated complete death of *P. americana* and increased mortality rate in both stages of *P. americana*, especially the final-instar nymphs. Thus, for development of a cockroach control product, the concentration of extract should be increased. However, the solubility of dichloromethane extract in large amounts with a safe and suitable solvent, including stability test of the extract and resistance of *P. americana*, requires further study.

Didehydrostemofoline, a high potency insecticidal compound [26] found in the dichloromethane extract, presented the ability of penetration and distribution from the waxy integument of *P. americana* to other parts of the body. Results showed that dichloromethane extract and didehydrostemofoline could be used as insecticidal ingredients in spray and aerosol formulations as well as bait formulations. The dosage forms are easy and convenient to use. This research was the first report on the adulticidal and nymphicidal activities and distribution of didehydrostemofoline in the tissue of *P. americana*. This basic knowledge can lead to the continuous future development of cockroach control.

5. Conclusions

Natural products are sources of insecticides. Dichloromethane *S. collinsiae* root extract containing a large amount of didehydrostemofoline displayed nymphicidal and adulticidal activities against *P. americana* via contact administration as a new route of this extract. The extract can be used as an active ingredient in the form of spray and aerosol. Didehydrostemofoline penetrated through the cuticle, was absorbed and distributed to tissues and finally bound with the target site, with distribution detectable using the MALDI IMS technique. Signs of toxicity such as shaking body, tremors, motionlessness and swollen abdomen occurred in *P. americana* contacting dichloromethane extract, similar to *P. americana* ingesting bait containing dichloromethane extract because the same phytochemicals or pharmacophores acted on the same biological target site. Didehydrostemofoline caused signs of toxicity and death in *P. americana* contacting the dichloromethane extract. The didehydrostemofoline penetrated through the pore canals, dermal ducts or spiracles. According to the Role of 5, didehydrostemofoline can be used as an active ingredient in aerosol and spray formulations for cockroach control. However, toxicity testing in mammals requires rigorous future investigation.

Author Contributions: Conceptualization, A.S.; methodology, A.S.; software, A.S. and K.O.; validation, A.S. and K.O.; formal analysis, A.S. and K.O.; investigation, A.S., K.O. and J.S; resources, A.S. and A.P.; data curation, A.S. and K.O.; writing—original draft preparation, A.S.; writing—review and editing, A.S.; visualization, A.S. and K.O.; supervision, R.S. and J.R.; project administration, A.S.; funding acquisition, A.S.

Funding: This research was funded by Faculty of Pharmacy, Thammasat University Research Fund under the Specific Field Research Scholar: Research Unit in Phytochemistry and Natural products, Contact No. Pharm TU-S 1/2019 and Faculty of Pharmacy, Thammasat University Research Fund under the Specific Field Research Scholar: Research Unit in Phytochemistry and Natural products, Contact No. Pharm TU-S 1/2021.

Institutional Review Board Statement: The study was approved by Ethics Committee and accordance with ethical principles and guidelines for the use of animals provided by the National Research Council of Thailand. All experiments were performed in accordance with protocols approved by

the Animal care and Use Committee of Thammasat University: Protocol No. 005/2020 and the Animal care and Use Committee of Faculty of Tropical Medicine, Mahidol University: Protocol No. 001/2021 Certificate No. FTM-ACUC 005/2021.

Data Availability Statement: The supporting data in this research are available from the corresponding author upon request.

Acknowledgments: We have to thank Assoc. Prof. Dr. Rathapon Asasutjarit and Asst. Prof. Dr. Patchara Sriwichai and Nattanan Timrod for supporting and facility.

Conflicts of Interest: The authors declare no conflict of interest.

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