Research article

Oral toxicity of various *Stemona collinsiae* crude extracts against nymph and adult stages of American cockroach, *Periplaneta americana* (Dictyoptera: Blattodea)

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ABSTRACT

Stemona collinsiae exhibits insecticidal resistance against various pests and insect vectors. However, insecticidal activity of *S. collinsiae* roots has not been tested for some insect vectors, including the American cockroach, *Periplaneta americana*. The synanthropic insect *P. americana* is a reservoir of pathogenic and non-pathogenic microorganisms and a cause of infectious diseases and cockroach allergy. This important vector transmits microorganisms to animals and humans to cause vector-borne diseases. This research involved detection of the nymphicidal and adulticidal activities of *S. collinsiae* root extracts against *P. americana* through oral administration. The effects of hexane, dichloromethane, ethanol, and water crude extracts were tested on final instar nymphs and adult *P. americana*. After *P. americana* ingested bait containing hexane and dichloromethane crude extracts, signs of toxicity occurred, such as hind leg shaking, whole-body tremor, immobility, abdomen swelling, and death. At 48 h, the nymphs and adult *P. americana* that ingested dichloromethane crude extract-containing bait showed corrected mortality of 65%–100% and 20%–100%, respectively. Whereas none of the nymphs and adult *P. americana* that ingested the water crude extract-containing bait died (0% corrected mortality). When we dissected alimentary canals of the dead *P. americana* that had ingested dichloromethane and hexane crude extract-containing baits, the foreguts were found to be swollen. TLC analysis showed the dichloromethane and hexane crude extracts contained the alkaloid didehydrostemofoline and unknown fluorescent substances. Phytochemicals from crude extracts were detected in extracts of dissected alimentary canals using thin-layer chromatography, and didehydrostemofoline alkaloid and unknown fluorescent substances were found in cockroaches that ingested dichloromethane- and hexane-containing baits. The cause of death of *P. americana* may be attributed to alkaloids and synergistic effects of other substances in *S. collinsiae* root extract. Mechanisms of action might include several pathways involved in nervous system function. Thus, dichloromethane and hexane crude extracts can be developed as alternative active ingredients in a natural insecticide for cockroach control.

1. Introduction

Periplaneta americana is an omnivorous, synanthropic insect and an important insect vector. *P. americana* is found at approximately 60.9% in 14 provinces of Thailand (Tawatsin et al., 2001), and is globally distributed, including in Korea (Lee et al., 2003), China (Chompoosri et al., 2004), and Iran (Kassiri et al., 2014). *P. americana* prefers a warm climate with high humidity and prefers living in dark places. Cockroaches such as *P. americana* are indicators of poor hygiene and are often found in indoor and outdoor facilities, such as kitchens, restaurants, toilets, and sewers (Chompoosri et al., 2004; Sriwichai et al., 2002; Lee et al., 2003; Tawatsin et al., 2001). The cockroach's alimentary cavity and salivary glands are reservoirs for the habitation and propagation of both non-pathogenic and pathogenic microorganisms (Falsone et al., 2017; Tinker and Otteson, 2016; Gijzen and Barugahare, 1992; Bracke et al., 1979); thus, *P. americana* can carry several vector-borne diseases, such as food poisoning, parasitic diseases, and typhoid disease. Microorganisms such as *Klebsiella, Pseudomonas, Escherichia coli, Proteus, Salmonella,*

Staphylococcus, Streptococcus, Strongyloides stercoralis, Ascaris lumbricoides, Trichuris trichiura, Isospora belli, Entamoeba histolytica, Entamoeba coli, and *Iodamoeba butschlii* (Kassiri et al., 2014; Malik et al., 2013; Chamavit et al., 2011; Pai et al., 2005) can be transmitted to humans and animals via mechanical and biological transmission (Pai et al., 2003; Tatfeng et al., 2005; Suntaravitun and Domaikaw, 2018; Zahraei-Ramazani et al., 2018: Malik et al., 2013; El-Sherbini andGneidy, 2012; Chamavit et al., 2011). Cockroaches mediate mechanical transmission when they climb over waste and sewage, which are sources of pathogenic and non-pathogenic microorganisms. Microorganisms, parasites, and their eggs can opportunistically attach to the skin, hair, legs, and wings of the cockroach and be transferred to human or animal foodstuffs by the insect when it is foraging, resulting in the spread of infectious diseases. In other cases, microorganisms can contaminate human and animal diets via secretions from cockroach salivary glands, i.e., biological transmission. *P. americana* is also a transport vector of *Toxoplasma gondii* (Chinchilla et al., 1994), the pathogenic protozoan that causes toxoplasmosis (Dubey, 2008). Cats, the definitive hosts, ingest *T. gondii* by eating infected raw meat, rodents, or arthropods, such as house flies and cockroaches (Hill and Dubey, 2002), and excrete the oocytes of *T. gondii* in their faeces. The faeces are then ingested by cockroaches (Wallace, 1972). When a pregnant woman comes into contact with oocytes via fecal-oral administration or exposure to cat feces, *T. gondii* may be transmitted from the mother to the unborn child, who is at risk of serious symptoms such as blindness, mental disability, hydrocephalus, chorioretinitis, intracerebral calcification, and physical disability (Dubey, 2008; Halonen and Weiss, 2013). Thus, the elimination of cockroaches can decrease the rate of transmission of *T. gondii* and other pathogenic microorganisms from cockroaches to animals and humans.

A well-known non-infectious disease associated with cockroaches is cockroach allergy (Srisuwatchari et al., 2020; Nasirian, 2017; Patel and Meher, 2016; Sookrung et al., 2014; Arruda et al., 2001; Schou et al., 1990). The exoskeleton, whole body, secretions, feces, and dust particles from dead cockroaches are all sources of allergens (Patel and Meher, 2016). The main allergen Per 1a has been extracted and isolated from the midgut and feces of *P. americana* (Sookrung et al., 2014), and other allergens have been found, including Per 2a, Per 3a, Per 6a, Per 7a, Per 9a, and Per 10a; these allergens may cause indoor allergy and asthma in infants, children, and adults (Arruda et al., 2001). After a patient receives allergens via contact and inhalation, the allergens react with immunoglobulin E (IgE) and non-IgE types, leading to the secretion of cytokines and chemokines. Allergic inflammation occurs, during which white blood cells are increasingly induced and move into the inflammation site, while mast cells release histamine. Finally, allergic symptoms such as a red rash, sneezing, rhinorrhea, allergic rhinitis, airway inflammation, bronchospasm, wheezing, and asthma appear (Srisuwatchari et al., 2020; Patel and Meher, 2016).

Therefore, eliminating cockroaches and terminating their life cycle, for instance via mechanical traps and chemical insecticides, is an important strategy for controlling and preventing cockroach vector-borne diseases. Insects can receive chemical insecticides via ingestion, contact, and inhalation. The ingestion pathway, usually using oral bait, is convenient, ready to use, and simple for insect control. The bait does not widely diffuse in air and good insect control should have a low ecological impact (Revay et al., 2015). The toxic bait is contained in closed containers which are safe for humans and pets. After insects eat the toxic bait, the toxic insecticides are absorbed and distributed to the tissue (Champman, 1998). Irregular symptoms occur, leading to the death of the insect. Insecticidal activity via the ingestion route is an interesting basic route and toxic bait products are available in the market. Accordingly, the bait method was used in this research. Natural chemicals from insecticidal plants are sources of synthetic insecticides such as pyrethroids and imidacloprid. In Thailand, there is a high diversity of insecticidal plants, including *Stemona collinsiae* (Stemonaceae), which has been traditionally used as an insecticide and insect repellant to protect food. Furthermore, the root extract has been used as a pesticide for eliminating beetles and pests in pepper plantations (Inthachub and

Duyfjes, 2011). Recent scientific reports revealed that *S. collinsiae* root extract can be used to kill pests such as *Plutella xylostella* (Jiwajinda et al., 2001; Phattharaphan et al., 2010), *Spodoptera exigua* (Brem et al., 2002), and insect vectors such as *Parasarcophaga ruficornis* (Sakulpanich et al., 2017) and *Chrysomya megacephala* (Sakulpanich et al., 2016), but its insecticidal activity against *P. americana* has never been reported. Thus, in this research, we studied the nymphicidal and adulticidal activities of various *S. collinsiae* root extracts against final instar nymph and adult stage *P. americana* via oral administration. The crude extracts were produced using a sequential reflux extraction method, and phytochemicals in each crude extract were detected with thin-layer chromatography (TLC). Bait mixed with each crude extract was fed to *P. americana* to observe the resulting mortality and signs of toxicity, including irregularities of the internal organs.

2. Materials and methods

2.1. Plant materials

2.1.1. Stemona collinsiae preparation

Roots of *S. collinsiae* were harvested from Ubon Ratchathani, Thailand, from December 2018 to January 2019, and the roots and aerial parts were arranged as herbarium for identification. The roots were first cleaned with tap water and then dried using an electric fan to eliminate excess water on the surface of the roots and cut up into small pieces. Next, they were dried in a hot air oven at 55 ± 1 °C for 72 h. The small dry pieces of root were ground and sieved using a #30 mesh sieve, and the powdered root was used in further reflux extraction processes.

The herbarium of *S. collinsiae* root and aerial parts was 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.

2.1.2. Preparation of hexane, dichloromethane, ethanol, and water crude extracts using sequentially reflux extraction method (modified from Sakulpanich et al., 2017)

Hexane, dichloromethane, ethanol, and purified water were used as extractants. For hexane extraction, powdered root (300 g) was placed in a round-bottom flask, 1000 ml of hexane was added, and the top of the flask was covered with a cold condenser. The flask was soaked in a 60–70 °C water bath for 1 h, then the liquid extract was passed through a filter paper and the filtrate collected in a bottle and protected from light. The filtrate was tested for alkaloids by analyzing a 2 ml aliquot using thin-layer chromatography (TLC) and Dragendorff's spray reagent. A new aliquot of hexane was added into the extraction residue, and the flask was re-immersed in the water bath for 1 h. The extraction and filtration process were repeated until all alkaloids were exhaustively extracted from the extraction residue. The complete extraction was confirmed by TLC and Dragendorff's spray reagent. The collected filtrates were concentrated using a rotary evaporator under reduced pressure at 40 ± 1 °C. Then, the hexane crude extract was dried on a water bath at 70 ± 1 °C, poured into a tightly sealed glass container, protected from light, and kept in a refrigerator at 4 °C.

For dichloromethane, ethanol, and water extraction, the extraction process was repeated using the same steps as described in the hexane extraction. The dichloromethane, ethanol, and water were changed when alkaloid-testing showed a negative result in the final filtrate. The water crude extract was dried using lyophilization.

2.2. Ethical consideration statement

The animal protocol was established under ethical principles and guidelines for the use of animals provided by the National Research Council of Thailand and all animal experiments were performed in accordance with the 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.

2.3. Periplaneta americana rearing

Periplaneta americana were collected from Ratchaburi, Thailand. The cockroach species were identified by an Entomologist, Yudthana Samung, who works in Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University. The species identification was performed following the handbook of domiciliary cockroach species in Thailand (Asahina, 1983). *P. americana* were reared by Anon Phayakkaphon who works in the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University; they were provided with dry cat food (Purina® Friskies® for adult cats, Nestlé, Thailand) and water. 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 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 h dark: light cycle. Final instar nymphs and adult *P. americana* were used for oral toxicity tests using the bait method (Seanmanot et al., 2018; Thavara et al., 2007).

2.4. Oral toxicity based on bait method

2.4.1. Bait preparation

Each type of crude extract was homogenously mixed with glucose. The range of concentration, 1–50% w/w was tested. Two-fold serial dilutions were prepared (6 concentrations per two-fold dilution). The range of concentration of the crude extract, which produced the range of corrected mortality between 10-90% (Finney, 1971), was selected for calculating LC_{50}. Finally, 3 g of each concentration of the bait was weighed and placed in a disposable plastic Petri dish (90 × 15 mm) (สำนักงานคณะกรรมการอาหารและยา, 2538).

2.4.2. Bioassay

Bioassay (modified from Ko et al., 2016; Anikwe et al., 2014; Srinivasan et al., 2005; ประกาศสำนักงานคณะกรรมการอาหารและยา, 2538).

A group of 10 final instar nymphs (unsexed) and a group of 10 adult *P. americana* (mixed sexes) were used in the test. A cup of water was placed in the testing box. The *P. americana* were released into the box and starved for 24 h before testing. After 24 h, the bait was placed in the box, and signs of toxicity and mortality were observed at 1, 2, 4, 12, 24, 48, and 72 h. The 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. *P. americana* in the negative control group received only glucose. All experiments were repeatedly performed for three replications.

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2.5. Comparison of the dissected alimentary canals from the extract-treated group and negative control group

The 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. The 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 negative control group.

The images of *P. americana* and the dissected alimentary canal were taken using a Canon EOS 500D digital SLR camera (Canon, Japan). The SNZ745T stereomicroscope 10x (Nikon, China) with microscope camera MDX503 and iWorks software (Lanoptik Technologies Ltd., China) was used for enlarging the images.

2.6. Determination of alkaloids in crude extracts and dissected alimentary canal using TLC

2.6.1. Preparation of hexane, dichloromethane, ethanol, and water crude extract solutions

The hexane, dichloromethane, ethanol, and water extract solutions were prepared at a concentration of 1 mg/ml. The hexane and dichloromethane crude extracts were dissolved in dichloromethane, whereas the ethanol and water crude extracts were dissolved in 70% ethanolic solvent. In the case of water crude extract, a sonication bath was used for dissolving.

2.6.2. Preparation of dichloromethane alimentary canal extracts

Didehydrostemofoline is very soluble in dichloromethane; thus, dichloromethane was selected to extract the didehydrostemofoline from the dissected alimentary canals. The dissected alimentary canals of the dead nymph and adult *P. americana* in the treated groups and negative control group were soaked in dichloromethane. Dichloromethane (1 mL) was added to a container with the dissected alimentary canal, which was then shaken using a vortex for 3 min, and the dichloromethane liquid extract was pipetted into a new container. A new aliquot of dichloromethane was added to the container and shaken for 3 min, and the extraction was repeated three times. The collected dichloromethane liquid extract of the alimentary canal was dried using a rotary evaporator under reduced pressure and at 40 °C. Dichloromethane (1 mL) was added to the dry residue, and the alkaloids in the dichloromethane alimentary canal extract solutions were tested with TLC.

2.6.3. TLC method

Amounts of 20 μL of the dichloromethane alimentary canal extract solution, 10 μL of didehydrostemofoline, 10 μL of hexane, dichloromethane, and water crude extract solution, and 20 μL of ethanol crude extract solution were spotted onto a silica gel GF254 TLC plate (Merck, Germany) by Linomat 5 applicator (Camag®, Switzerland). The TLC plate was developed in a TLC tank containing a mixture of dichloromethane, methanol, water, and 10% NH_4OH in the ratio 70: 25: 5: 0.1. The developed TLC plate was dried and detected under visible and UV light at 254 nm and 366 nm, respectively. Then, the TLC was sprayed with Dragendorff's reagent to detect the alkaloids. The development of an orange band indicated the presence of an alkaloid. TLC images were taken and recorded by TLC Visualizer (Camag®, Switzerland) and the TLC performance was controlled by WinCats software (Camag®, Switzerland). TLC was performed by the authors and was performed repeatedly for three replications.

2.7. Parameter and statistical analysis

The percentage of observed mortality was calculated. The data were corrected using Abbott's formula (Abbott, 1925). The percentages of corrected mortality were calculated and shown as mean ± SD or range as min-max. Signs of toxicity, morphological changes and abnormal behaviour were observed. The onset of action and time to death were recorded and compared with the negative control group. The severity level of morphological and behaviour changes including signs of toxicity were considered and divided into groups. The percentage of the

P. americana in each group was calculated. Probit analysis using the Probit analysis program, March 1987 version (Raymond, 1985), was performed to calculate the median lethal concentrations (LC_{50}) at a 95% confidence limit of upper (UCL) and lower (LCL) confidence limits. Results with *p*-value < 0.05 are considered statistically significant.

3. Results

3.1. Oral toxicity based on bait method

Each type of extract resulted in different mortality percentages and nymphicidal and adulticidal activity levels. Forty-eight hours after treatment of the final instar nymphs with baits containing hexane, dichloromethane, ethanol, or water crude extract, we observed percentage corrected mortalities of 0%–30%, 65%–100%, 7%–13%, and 0%–0% respectively. The adult *P. americana* at 48 h after treatment with the baits containing hexane, dichloromethane, ethanol, or water crude extract displayed percentage corrected mortalities of 11%–54%, 20%–100%, 0%–40%, and 0%–0%, respectively. The mortality of *P. americana* that ingested the ethanol crude extract was less than those that consumed the dichloromethane and hexane extract baits. When the dichloromethane and hexane extract baits were suddenly placed in the test boxes, *P. americana* retreated. After 15–30 min, a few *P. americana* gradually approached and ate the bait, and 48–72 h after placing the bait, all *P. americana* that ingested the bait had died with a swollen abdomen. The baits containing the highest concentrations of dichloromethane and hexane extracts showed the most potent repellent effect. *P. americana* ate the bait mixed with the low-concentration ethanol crude extract and showed symptoms of leg shaking and body tremors, but 48 h later, the symptoms had disappeared and no dead *P. americana* were seen. Both the final instar nymph and adult *P. americana* that received the dichloromethane crude extract displayed the highest percentage corrected mortality. All *P. americana* in the water crude extract-treated group and in the negative control group (0%–0% of corrected mortality) survived and did not show any signs of toxicity.

All *P. americana* that ingested the bait containing hexane and dichloromethane crude extracts, and some that consumed the ethanol crude extract, showed toxicity symptoms, such as excited movement, body elevation, hind leg shaking, whole-body tremors, immobility, and abdomen swelling. Compared with the *P. americana* in the negative control group (Figure 1A), the *P. americana* that ingested the dichloromethane and hexane crude extract-containing baits showed clear abdomen swelling (Figure 1B). At the highest concentration (50% w/w) of each crude extract, the onset time of the excited movement and shaking symptoms appeared at 13–15 min after eating the bait containing dichloromethane crude extract, which occurred faster than the ethanol crude extracts (34–40 min). At 1–3 h after consuming the bait containing the dichloromethane crude extract, the swollen abdomen occurred. The shortest duration of time-to-death was 24 h which was found in the *P. americana* in the dichloromethane extract-treated group. At 24–72 h, the majority of *P. americana* in the ethanol extract treated group had not died, but they presented immobility.

Severe effects were found, such as inflating of the peritoneum, which led to a tear of the integument (1%) (Figure 2A) or leakage of adipose tissue from the intersegmental membrane (1%) (Figure 2B). The protrusion of anal organs (Figure 2C) occurred more frequently than both of the severe effects. The inflating of the peritoneum and leaking of adipose tissue happened in some *P. americana* that had repeatedly ingested the toxic bait containing low concentrations (1.0% w/w–2.5% w/w) or ate the bait containing dichloromethane crude extract at the high concentration (50% w/w).

Three main signs of toxicity that developed after oral administration represented three levels of symptom severity, from most to least severe: (1) swollen abdomen, (2) apathy and absence of movement, (3) defensive behaviour and rapid locomotion. The percentage of *P. americana* that displayed each sign is presented in Table 1. After *P. americana* ate the bait mixed with dichloromethane, hexane, and ethanol crude extracts, there
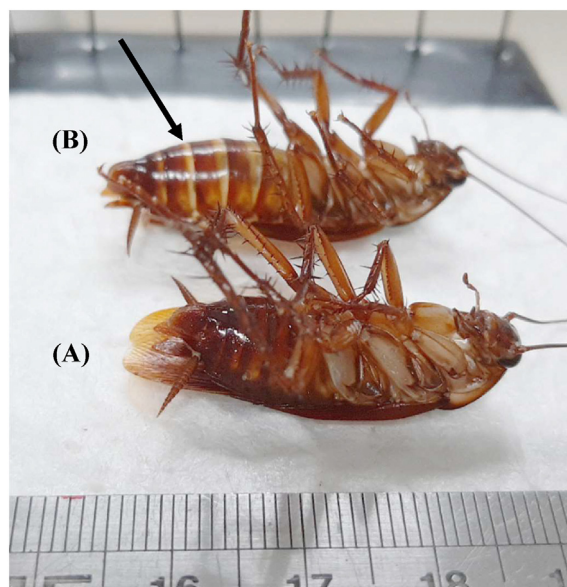

Figure 1. Comparison between (A) the adult *P. americana* in the negative control group and (B) the adult *P. americana* receiving the dichloromethane crude extract and clearly showed the abdomen swelling (black arrow).

was sequentially apathy and motionlessness, followed by swelling of the abdomen, and those showing a swollen abdomen usually died within 72 h. The symptom of abdomen swelling was an irreversible effect.

At 24 h, the *P. americana*, ingesting the dichloromethane crude extract, showed the highest percentage of the swelling abdomen (35.0–100.0% in final instar nymph and 10.0–85.0% in the adult stage) and the lowest percentage of defence effect and fast movement (0.0%). At 48 h, the number of *P. americana,* showing apathy and motionlessness, decreased (0.0–15.0% in final instar nymph and 20.0–80.0% in the adult stage) while the percentage of the swelling abdomen increased (50.0–100.0% in final instar nymph and 20.0–95.0% in the adult stage). At 24 and 48 h, the ethanol crude extract predominantly produced the highest percentage of apathy and motionlessness (20.0–88.9% in final instar nymph and 10.0–70.0% in the adult stage) while the percentage of the swelling abdomen was lowest, compared with the dichloromethane and hexane crude extract treated groups. The *P. americana* eating the water crude extract and the *P. americana* in the negative control group eating only the glucose did not show any sign of toxicity (0% of swelling abdomen, apathy and motionlessness) and still showed fast movement and reactions to stimulants (100% of defence effect with fast movement).

3.2. Comparison of the dissected alimentary canals in the extract-killed *P. americana* and the *P. americana* in the negative control group

After the *P. americana* in the hexane- and dichloromethane-treated groups died with swollen abdomens, they were dissected to study the internal organs. We found that their foreguts were swollen compared with those in the water crude extract-treated and negative control groups, which showed no swelling (Figure 3). Some *P. americana* in the ethanol-treated group with swollen abdomens were dissected, and the foregut was found to be swollen in a similar manner to that of the *P. americana* in the dichloromethane- and hexane-treated groups. Black digested food was observed in the foreguts of the ethanol-, dichloromethane-, and hexane-treated groups but was not seen in the foreguts of the negative control group, which ate only glucose (Figure 3).

3.3. Determination of alkaloids in the crude extracts and the dissected alimentary canal using TLC method

The orange bands appeared after spraying the TLC plate with Dragendorff's spray reagent (Figure 4C), which indicated the presence of
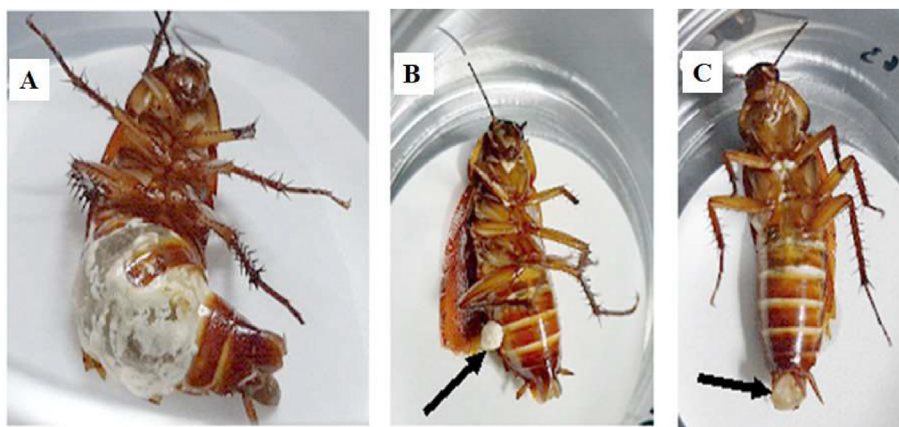
Figure 2. The severe effect in the adult *P. americana* causing from the ingestion of toxic bait. (A) inflating of peritoneum and tearing of exoskeleton which happened with *P. americana*, eating repeatedly the dichloromethane bait, and (B) leaking of adipose tissue (black arrow) and (C) the protruded anus part (black arrow) was frequently occurred in the hexane and dichloromethane crude extract-treated group.

Table 1. The percentage of the signs of toxicity divided into three levels in each group of *P. americana* ingesting the different extracts, observed at 24 and 48 h.

Group of *P. americana*	Time of observation	The percentage of *P. americana*, observed at 24th and 48th hour					
		Final-instar nymph			Adult *P. americana*		
		Swollen abdomen	Apathy and absence of movement	Defense behavior and rapid locomotion	Swollen abdomen	Apathy and absence of movement	Defense behavior and rapid locomotion
Hexane treated group	24th hour	11.1–55.0 (29.2 ± 1.7)	25.0–77.8 (56.8 ± 2.3)	0.0–20.0 (11.0 ± 0.1)	16.7–60.0 (20.7 ± 1.5)	57.2–88.9 (71.6 ± 1.4)	0.0–10.0 (4.5 ± 0.2)
	48th hour	21.7–60.0 (39.3 ± 1.6)	25.0–73.3 (50.7 ± 2.0)	0.0–5.0 (2.7 ± 0.2)	16.7–60.0 (38.0 ± 1.9)	42.5–80.7 (61.8 ± 1.6)	0.0–0.0 (0.0 ± 0.0)
Dichloromethane treated group	24th hour	35.0–100.0 (74.0 ± 2.8)	0.0–30.0 (16.8 ± 1.2)	0.0–0.0 (0.0 ± 0.0)	10.0–85.0 (31.5 ± 2.9)	10.0–90.0 (57.2 ± 2.9)	0.0–20.0 (8.4 ± 0.7)
	48th hour	50.0–100.0 (84.0 ± 2.7)	0.0–15.0 (10.0 ± 0.8)	0.0 ± 0.0 (0.0 ± 0.0)	20.0–95.0 (33.0 ± 2.2)	20.0–80.0 (54.0 ± 2.2)	0.0–0.0 (0.0 ± 0.0)
Ethanol treated group	24th hour	0.0–33.3 (17.2 ± 1.4)	20.0–88.9 (60.8 ± 2.9)	0.0–80.0 (37.0 ± 3.2)	0.0–30.0 (16.0 ± 1.3)	10.0–70.0 (42.0 ± 2.6)	0.0–60.0 (30.0 ± 2.5)
	48th hour	0.0–20.0 (8.4 ± 0.7)	20.0–88.9 (56.4 ± 2.5)	0.0–80.0 (40.5 ± 2.8)	0.0–30.0 (12.0 ± 1.3)	10.0–70.0 (36.0 ± 2.6)	0.0–60.0 (30.0 ± 2.5)
Water treated group	24th hour	0.0–0.0 (0.0 ± 0.0)	0.0–0.0 (0.0 ± 0.0)	100 (100.0 ± 0.0)	0.0 ± 0.0 (0.0 ± 0.0)	0.0 ± 0.0 (0.0 ± 0.0)	100 (100.0 ± 0.0)
	48th hour	0.0–0.0 (0.0 ± 0.0)	0.0–0.0 (0.0 ± 0.0	100 (100.0 ± 0.0)	0.0 ± 0.0 (0.0 ± 0.0)	0.0 ± 0.0 (0.0 ± 0.0)	100 (100.0 ± 0.0)
Glucose treated group (Negative control)	24th hour	0.0–0.0 (0.0 ± 0.0)	0.0–0.0 (0.0 ± 0.0)	100 (100.0 ± 0.0)	0.0 ± 0.0 (0.0 ± 0.0)	0.0 ± 0.0 (0.0 ± 0.0)	100 (100.0 ± 0.0)
	48th hour	0.0–0.0 (0.0 ± 0.0)	0.0–0.0 (0.0 ± 0.0)	100 (100.0 ± 0.0)	0.0 ± 0.0 (0.0 ± 0.0)	0.0 ± 0.0 (0.0 ± 0.0)	100 (100.0 ± 0.0)

Range presented in range of min-max.
(…) presented as mean ± SD.

alkaloids. Quenching bands were seen under light at 254 nm (Figure 4A). Orange and quenching bands were seen at the same position ($R_f = 0.41$) in the didehydrostemofoline reference substance (Row No. 9), hexane crude extract (Row No. 6), and dichloromethane crude extract (Row No. 7), including the extracts of the dissected alimentary canals from the adult *P. americana* (Row No. 2 and 3) and final instar nymphs (Row No. 12 and 13) that ingested the hexane and dichloromethane crude extract-containing baits.

An orange band corresponding to didehydrostemofoline alkaloid was apparent in the dichloromethane crude extract and *P. americana* that ingested bait containing hexane and dichloromethane crude extracts, while the water crude extract did not show this band. All extracts from dissected alimentary cavities exhibited the didehydrostemofoline band at

$R_f = 0.41$, except those of the final instar nymph and adult *P. americana* that ate the bait containing the water crude extract (Row No. 5 and 15). The highest concentration of didehydrostemofoline was found in the extract from the alimentary canal of adult *P. americana* that consumed dichloromethane bait (Row No. 3), followed by that of the final instar nymphs that ingested the dichloromethane bait (Row No.13).

Under light at 366 nm (Figure 4B), blue and purple fluorescent bands appeared at $R_f = 0.73$ and $R_f = 0.75$, respectively. Both fluorescent bands appeared in the row containing the dichloromethane crude extract (Row No. 7) and the alimentary canal extracts of adult *P. americana* (Row No. 3) and final instar nymphs (Row No. 13) that ingested the dichloromethane crude extract. The highest fluorescence intensity occurred for the alimentary canal extract from the adult *P. americana* that ate the
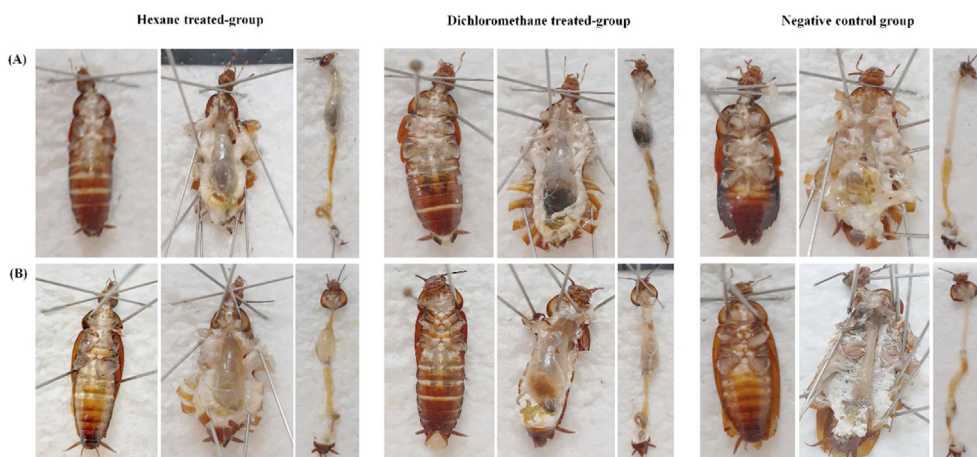
Figure 3. The dissected alimentary canals of (A) the final instar nymphs and (B) the adult *P. americana* in the hexane crude extract-treated group, the dichloromethane crude extract-treated group and the negative control group receiving only glucose.
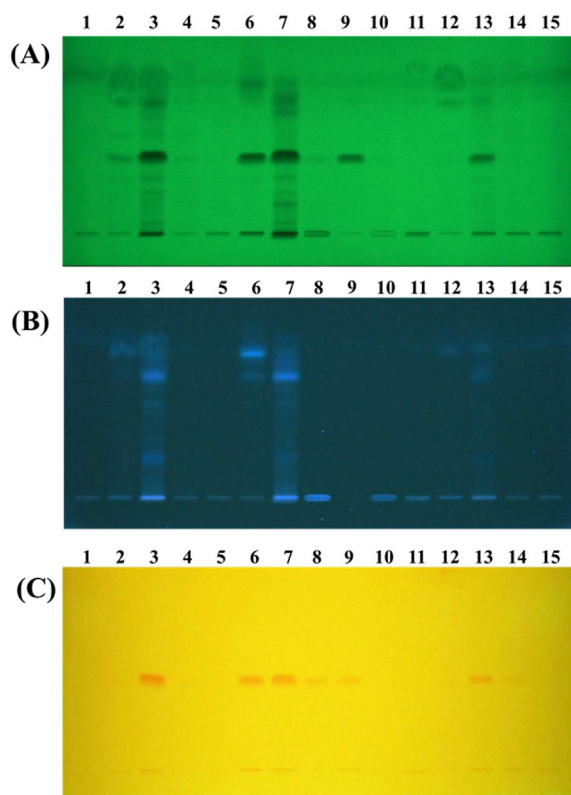

Figure 4. TLC pattern of the crude extracts and the extracts from the dissected alimentary canals which was observed under (A) UV 254 nm, (B) 366 nm and (C) after sprayed with dragendorff's spray reagent. 1 = Adult *P. americana* ingesting glucose. 2 = Adult *P. americana* ingesting hexane bait. 3 = Adult *P. americana* ingesting dichloromethane bait. 4 = Adult *P. americana* ingesting ethanol bait. 5 = Adult *P. americana* ingesting water bait. 6 = Hexane crude extract. 7 = Dichloromethane crude extract. 8 = Ethanol crude extract. 9 = Didehydrostemofoline reference substance. 10 = Water crude extract. 11 = Final-instar nymph ingesting glucose. 12 = Final-instar nymph ingesting hexane bait. 13 = Final-instar nymph ingesting dichloromethane bait. 14 = Final-instar nymph ingesting ethanol bait. 15 = Final-instar nymph ingesting water bait.

dichloromethane crude extract (Row No. 3), and the second highest intensity was seen for the gut extract of final instar nymphs that consumed the dichloromethane crude extract (Row No. 13).

4. Discussion

Stemona collinsiae and crude extracts including alkaloids such as didehydrostemofoline, stemofoline, and hydroxystemofoline (Kongkiat-paiboon et al., 2011; Schinnerl et al., 2007; Greger, 2006; Sastraruji et al., 2005; Seger et al., 2004; Pham et al., 2002; Jiwajinda et al., 2001) exhibit insecticidal activity against pests and important insect vectors (Sakul-panich et al., 2016, 2017; Phattharaphan et al., 2010; Vo et al., 2010; Brem et al., 2002; Jiwajinda et al., 2001), but *P. americana* has never been tested with *S. collinsiae*. This study was the first report that presented the nymphicidal and adulticidal activities of *S. collinsiae* root extracts against *P. americana*. The highest didehydrostemofoline content was in the dichloromethane crude extract and was followed by hexane and ethanol crude extracts while the absence of didehydrostemofoline was found in the water crude extract. The dichloromethane crude extract presented the highest potency against *P. americana* followed by the hexane and ethanol crude extracts. All *P. americana* in the groups that received water crude extract and only the glucose survived. The least amount of dide-hydrostemofoline was found in the ethanol crude extract because the didehydrostemofoline was not exhaustively extracted in the previous extraction with dichloromethane extractant. The lowest didehy-drostemofoline content in the ethanol crude extract slightly affected the mortality rate in the *P. americana*. Therefore, didehydrostemofoline was the cause of death in the *P. americana* that ingested the toxic baits, especially the dichloromethane crude extract-containing bait. Interest-ingly, the phytochemicals possessed blue and purple fluorescent bands on TLC, which were most prominent in the dichloromethane crude extract, including extracts from the dissected alimentary cavity of the final instar nymph and adult *P. americana* ingesting the bait containing dichloro-methane crude extract. Thus, it is possible that the unknown blue and purple fluorescent phytochemicals were also the cause of death in the dichloromethane treated *P. americana*. These fluorescent phytochemicals and other phytochemicals, such as stilbenoids and rotenoid flavonoids, might synergize the insecticidal activity of the alkaloids. Recently, stil-benoids isolated from *Vitis vinifera* canes reportedly exhibited insecticidal activity against *Spodoptera littoralis* larvae (Pavela et al., 2017); however, its effects on *S. collinsiae* are unclear and should be investigated in further experiments.

The oral toxicities of the *S. collinsiae* root extracts were novel, as evident from the findings. Signs of toxicity were observed, including hind leg shaking, body tremors, undirected and fast movement, immobility, pa-ralysis, abdomen swelling, and finally death. The strongest toxicity signs were seen in *P. americana* that ate the dichloromethane crude extract-containing bait, and the strength was directly associated with the con-centrations of didehydrostemofoline and unknown fluorescent substances.

These toxicity symptoms are similar to those reported for nitromethylene heterocycle insecticides and neonicotinoids such as imidacloprid. The latter produces irregular and strong excitatory responses, such as abdominal quivering, wing flexing, uncontrollable preening, leg tremor, violent whole-body shaking, as well as depressive paralytic responses, such as prostration, immobility, paralysis (Tan et al., 2007; Schroeder and Flatum, 1984). Didehydrostemofoline containing pyrrolo[1,2-a]azepine (Schinnerl et al., 2007; Seger et al., 2004) or other alkaloids has some functional groups or pharmacophores similar to nitromethylene heterocycle insecticides and neonicotinoids comprising imidazolidine rings with electronegative atoms and N-containing cyclic/acyclic substituents (Schroeder and Flatum, 1984). Furthermore, alkaloids such as muscarine, tubocurarine, conitine, and nicotine generally act on the nervous and muscle systems. Thus, the insecticidal activity of *S. collinsiae* root extract is a result of alkaloids that induce neonicotinoid-like signs of toxicity.

The toxicity of didehydrostemofoline and stemofoline alkaloids which occurred from the inhibitory activity of acetylcholinesterase was reported (Kongkiatpaiboon et al., 2013). When considering other mechanisms behind the stimulation of nicotinic receptors and according to IRAC (IRAC, 2020), insect nicotinic acetylcholine receptor agonists and allosteric activators are of interest. Imidacloprid activates nicotinic acetylcholine receptor desensitized (nAChRD), while methyllicaconitine interacts with nicotinic acetylcholine receptor nondesensitized (nAChRN). Neonicotinoids, such as acetamiprid and clothianidin, are able to interact with α-bungarotoxin-insensitive nicotinic acetylcholine receptors both nAChR1 and nAChR2 to induce a biphasic current-voltage curve, whereas imidacloprid produces a monophasic current-voltage curve (Tan et al., 2007; Thany et al., 2007; Thany, 2009; Thany and Tricoire-Leignel, 2011). In comparison with this research, in terms of hyper-excitation, the cockroaches treated with the dichloromethane and hexane extracts in our study showed toxicity signs in the early stages, followed by lethargy. Because of the chemical structure-activity relationship, it is possible that the alkaloids within the *S. collinsiae* root extracts, especially the dichloromethane and hexane crude extracts, activate the nicotinic acetylcholine receptors, such as nAChRD and nAChRN, in the thoracic ganglia, or nAChR1 and nAChR2 in the DUM neurons. The mechanisms of action of the alkaloids isolated from *S. collinsiae* that interact with nicotinic acetylcholine receptors, and their structure-activity relationships, should be explored in further experiments.

Other insecticidal phytochemicals, such as stilbenoids (e.g., stemofurans A−K, dihydrostilbenes, stilbostemins A) and rotenoid flavonoids (e.g., stemonacetal, stemonal, and stemonone) (Zraunig et al., 2014; Pangkam and Chimsook, 2013; Pacher et al., 2002; Shiengthong et al., 1974), were found in *S. collinsiae* which exhibit inhibitory activity on mitochondrial complex I electron transport (IRAC, 2020). The *S. collinsiae* root extracts, especially the dichloromethane crude extracts, were found to comprise alkaloids and other unknown compounds but they might also contain rotenoids. The alkaloids, rotenoids, and unknown substances could produce synergistic effects and promote the insecticidal activity of *S. collinsiae* root extracts.

In this research, irregular and prominent swelling of the abdomen and foregut was surprisingly found in all dead *P. americana*. These symptoms were irreversible and important causes of death in *P. americana,* especially those that ate the bait mixed with the dichloromethane crude extract. The protrusion of the anal organ and leakage of adipose tissue were a result of the excess foregut swelling and the swollen foregut being pressed against the internal organs/adipose tissue. The swelling of the abdomen and foregut was not found in the negative control group or water crude extract group but the milder effect was noticed in the ethanol crude extract group. The swollen abdomen and foregut in *P. americana* that ingested the dichloromethane crude extract may have resulted from didyhydrostemofoline and other alkaloids which disturbed the function of neurotransmitters and the nervous system as well as crop hydrostatic pressure, transient pressure, and hemolymphic osmotic pressure which affected crop volume and crop emptying time because the muscles of the foregut and anterior midgut, including those involved in peristaltic movement, are controlled by the stomodeal nervous system, while the release of air from the crop during the feeding process is controlled by the pharyngeal and proventriculus nervous pathways. Pressure in the hemolymph influences the hydrostatic pressure gradient in the crop lumen and the anterior part of the midgut and leads to the opening of the proventricular valve (Davey and Treherne, 1964). Interference with the function of the foregut occurred consecutively. Moreover, crop emptying is regulated by the osmotic pressure of the hemolymph, which is controlled by dietary factors. At high hemolymphic osmotic pressure, the crop emptying time is prolonged (Champman, 1998), and the absorption of toxicants and the severity of toxicity increase. However, the actual cause of the abdomen and foregut swelling is uncertain.

Unexpectedly, the bait containing hexane and dichloromethane crude extracts as active ingredients showed cockroach-repellent properties, as both the final instar nymphs and adult *P. americana* retreated from these crude extracts, which affected the LC_{50} calculation. The relationship between the concentration of the crude extract and oral toxicity was dose-independent. Therefore, the LC_{50} values calculated in this research do not represent the actual values. In contrast, the ethanol and water crude extracts including glucose did not show repellent properties, and the starved *P. americana* ate the bait soon after it was placed in the boxes. Both the dichloromethane and hexane crude extract-containing baits, which possessed an odor and repellent activity, contained high concentrations of didehydrostemofoline and blue and purple fluorescent phytochemicals. Thus, we preliminarily presumed that the odor and repellent effect might be produced by the alkaloids or the unknown fluorescent substances. Likewise, repellent hetisine alkaloid was reported to repel *Tribolium casteneum* (Ulubelen et al., 2001). However, insecticidal compounds in the dichloromethane and hexane crude extracts should be isolated and the repellent compounds separated to remove the characteristic odor that disturbs *P. americana*, allowing bait containing only the isolated insecticidal compounds to be prepared and tested. The calculation of LC_{50} should subsequently be renewed to obtain the actual value.

Novelty of repellent activity from *S. collinsiae* crude extract based on the concentration was also found in this research. *P. americana* were obviously killed by *S. collinsiae* crude extract via oral administration using a bait form. *P. americana* has self-grooming behavior (Zhukovskaya, 2014). It is an omnivorous insect and opportunistic feeder (Bell and Adiyodi, 1982). It could ingest a residue of the crude extract attached to its antenna, foreleg, midleg, hindleg, wing, abdomen, corpse, exuviae, or other diet. Thus, aerosol was also a form of interest, but the suitable concentration, stability, and shelf-life of the insecticidal phytochemicals in the crude extract should be selected and detected. Besides, didehydrostemofoline, having a molecular mass less than 500, and lipophilicity, could penetrate from integument via pore canal and gland including a soft intersegmental membrane. It was absorbed and distributed to the target site. It was accumulated in the lipid tissue of *P. americana*. For the development of an aerosol formulation, contact toxicity should be tested for finding the evidence of efficacy of *S. collinsiae* against *P. americana* in the next experiment.

5. Conclusions

The dichloromethane and hexane crude extracts showed high toxicity to *P. americana*. The ethanol crude extract was less toxic, while the water crude extract was non-toxic to *P. americana*. The dichloromethane and hexane crude extracts killed the final instar nymph and adult *P. americana* and, thus, can terminate their life cycle and could be applied for insect control. The alkaloids found in *S. collinsiae* root extracts act as neurotoxins and cause irregular symptoms and death in *P. americana*. Didehydrostemofoline alkaloid is reported to possess acetylcholinesterase-inhibiting activity; however, other phytochemicals may also be responsible for the insecticidal activity, including rotenoids, flavonoids, and other unknown fluorescent substances, which may also synergistically promote the insecticidal properties. The mechanisms of actions of the

phytochemicals in *S. collinsiae* root extract might involve the nicotinic acetylcholine receptor modulator, nicotinic acetylcholine receptor allosteric modulator-site I, and mitochondrial complex I electron transport inhibitors. However, this needs further study because it was unclear which mechanisms were involved.

S. collinsiae roots contain an abundance of various insecticidal phytochemicals. This plant provides interesting raw materials for the development of alternative insecticides against the final instar nymph and adult *P. americana* via oral administration or in the form of a toxic bait or other form such as aerosol. This study found the repellent activity of the *S. collinsiae* hexane and dichloromethane crude extract. It can be used as a raw material in repellent products for repelling *P. americana*. The *S. collinsiae* root extract showed insecticidal and repellent activities against *P. americana* based on the concentration of the crude extract and the type of phytochemicals in the crude extract.

Declarations

Author contribution statement

Anon Phayakkaphon and Yudthana Samung: Contributed reagents, materials, analysis tools or data, Performed the experiments.

Preeyanate Dathong, Napasorn Ransibrahmanakul, Nontapat Sarovath: Performed the experiments.

Aurapa Sakulpanich: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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