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A plant-based extract mixture for controlling *Spodoptera litura* (Lepidoptera: Noctuidae)

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Abstract

Background: *Spodoptera litura* larvae are polyphagous insects that have become a significant pest in recent years. The spread of this pest has led to the continuous usage of insecticides on crops. Some plant extracts have been used as a mixture to control insect pests and improve productivity.

Methods: A plant-based mixture was mixed at a ratio of 1:1 v/v to demonstrate the effect on contact toxicity, feeding (no-choice test), and enzyme activities on *S. litura*. The active compounds of *P. retrofractum* and *A. calamus* were isolated by preparative thin-layer chromatography (PTLC).

Results: Our results showed that binary mixtures from *P. retrofractum* and *A. calamus* exhibit the highest contact toxicity and antifeedant activity at a 1:1 ratio of LD_{30}:LD_{10} dose (3.213 µg/larva *P. retrofractum* + 3.294 µg/larva *A. calamus*). The main active ingredient from each crude extract was (2*E*,4*E*,14*Z*)-*N*-isobutylicosa-2,4,14-trienamide from *P. retrofractum,* and beta-asarone and alpha-asarone from *A. calamus*. Additionally, *A. calamus* seems to be the synergistic compound. Some compound mixtures increased the glutathione-*S*-transferase activities in vivo; whereas, almost no significant differences in esterase activities were noted.

Conclusion: The results indicated that the ethanolic crude extracts of *P. retrofractum* and *A. calamus* mixtures could be used as the pesticidal compound and to develop a binary mixture formulation for controlling lepidopteran pests. However, the toxicity of this mixture to mammals needed to be explored before commercial development.

Keywords: *Spodoptera litura*, Plant extract mixture, Synergist, Detoxification enzymes, Antifeedant

Introduction

Spodoptera litura (Lepidoptera: Noctuidae) is a polyphagous insect pest that feeds on at least 87 plant species in over 40 plant families, including many vegetables, fruit, cotton, groundnut, chili, tobacco, castor, lady finger, cauliflower, and pulses, in many Asian countries, such as Thailand, China, Japan, India [1–3]. It has gradually become a significant insect pest in recent years [4]. The spread of this pest has led to the continuous usage of insecticides on crops. Pesticide residues impact the environment and people's health [5]. These results of insecticide usage have encouraged scientists to seek less hazardous chemicals and identify an alternative method for integrated pest management (IPM).

Botanical extract products have become more prominent in assessments of current and future pest control alternatives. They are biodegradable and ecologically safe and an important component of IPM programs [6]. For instance, numerous studies have focused on neem (*Azadirachta indica* A. Juss), and a number of studies demonstrate efficacy against a variety of pests [7, 8]. Its compound has numerous activities against insects, such as antifeedant, growth inhibition, growth regulation, reduced fecundity and sterility, and inhibition of protein synthesis, as well as toxic effects in a wide variety of insect taxa, including Lepidoptera [7, 8]. Plants comprise a source of novel chemical compounds that are used in

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medicine and other applications. Each plant contains many active compounds, such as terpenes, terpenoids alkaloids, steroids, phenols, and flavonoids [9], which are found in specific parts, i.e., leaves, flowers, stems, fruits, seeds, roots. Plant defense compounds typically result from a combination of these plant products and not as individual compounds.

A variety of documents suggest that complex mixtures would be more efficient, and synergistic effects have been reported [10, 11]. Singh et al. [12] demonstrated that thymol and α-terpineol synergized the impacts of both linalool and 1,8-cineole, but linalool with 1,8-cineole exhibited only an additive effect against *Chilo partellus*. *trans*-Anethole acted synergistically with thymol, citronellal and α-terpineol [13]. Consequently, the mixtures of plant compounds are also likely to be more durable against insects evolving resistance and developing behavioral desensitization.

This study was focused on the effect of six plant extracts, including *Acorus calamus* (Acorus calamus: Acorus calamus), *Alpinia galanga* (Zingiberaceae: Zingiberales), *Curcuma longa* (Zingiberaceae: Zingiberales), *Piper nigrum* (Piperaceae: Piperales), *Piper retrofractum* (Piperaceae: Piperales), and *Sphagneticola trilobata* (Asteraceae: Asterales), on *S. litura* control. These plant species are well known for their natural properties in the Thai traditional system of medicine or growth as a weed and have been reported to possess numerous types of biological activities.

Numerous anti-insect properties of the six plants involve toxic effects against many insects. Lee et al. [14] demonstrated the insecticidal effect of the methanol extract of *C. longa* rhizome on *Plutella xylostella* larvae. The hexane and ethanol extracts of rhizomes of *A. galanga* exhibit insecticidal activity against the fruit fly *Bactrocera dorsalis* when applied using a direct spray technique [15]. The essential rhizome oil of *A. calamus* showed a sterilizing effect against the eggs of *Sitophilus granarius*, *Sitophilus oryzae*, and *Culiosobruchus chinensis* [16] and larvicidal activity against *Culex quinquefasciatus* [17]. Upadhyay and Jaiswal found that 0.2 μl of *P. nigrum* oil significantly repelled *Tribolium castaneum* [18]. The dichloromethane extract of *P. nigrum* has pesticidal activity against *Callosobruchus maculates* and *Sitophilus zeamais* [19]. In addition to *P. nigrum*, *P. retrofractum*, which is in the same genus, also showed larvicidal activity against mosquito larvae *C. quinquefasciatus* [20]. Moreover, *S. trilobata* crude extracts have a larvicidal effect on *S. litura*, *S. exigua*, and *P. xylostella* larvae after topical application [21].

However, there is no research on the extract efficiency of the binary mixture in *S. litura*. The literature suggests that complex mixtures would be more effective than pure or only one crude extract [12, 13]. Thus, this research has the main goal of producing a plant-based mixture for control of *S. litura* with the possibility of increasing the control efficiency. Additionally, detoxification enzyme activities on treated *S. litura* were analyzed to search for the possibility of controlling this pest and examining the trends of resistance to this plant-based product in the future.

Materials and methods

Insect rearing

Spodoptera litura larvae used in this study were obtained from a laboratory colony maintained in the Animal Toxicology and Physiology Specialty Research Unit (ATPSRU), Department of Zoology, Faculty of Science, Kasetsart University. The culture was continuously maintained on an artificial diet (mixture of 240 g of green bean, 25 g of agar, 40 ml of mixed vitamin solution, 5 g of ascorbic acid, 40 ml of amoxicillin solution, 3 g of sorbic acid, 5 g of methylparaben, 20 g of yeast, 4 ml of 40% formalin and 1.41 l of water) in the insect-rearing room of the Department of Zoology, Faculty of Science, Kasetsart University, at 26 °C with 75% RH and a 16:8-h L:D photoperiod. Second and third instar larvae were used randomly for the treatment. All experimental procedures in this research were performed with the approval of an appropriate animal Ethics Committee of Kasetsart University, Thailand, under the reference number OACKU01059.

Plant materials and extraction methods

The rhizomes of *A. galanga*, *C. longa*, and *A. calamus*; the leaves and stem of *S. trilobata*; and the fruits of *P. nigrum* and *P. retrofractum* were obtained from Banphoromyen, Amphawa, Samut Songkhram province, Thailand. Each plant was rinsed with water to remove debris and air dried under shade. Dried plants were chopped finely to a powder. One kilogram of each powder sample was soaked in ethanol for 14 days. Each crude extract was filtered using a vacuum pump, dried by a rotary evaporator to obtain the solidified crude extracts and stored at 4 °C in a refrigerator until further processing.

Preliminary test of the contact toxicity bioassay for crude extract

Contact toxicity bioassays were performed with second instar larvae of *S. litura*. Each ethanolic crude extract was evaluated individually to determine efficacy levels upon topical application to the thorax region with various concentrations of extracts (2–140 μg/larva) using acetone as a carrier. Each second instar larva received 2 μl of extract per treatment for the thoracic region, and acetone alone served as the control. Thirty insects at each

concentration were used with five biological replicates. After treatment, larvae were maintained in the insect-rearing room and allowed to feed on an artificial diet. Mortality was recorded every day post-treatment. The median lethal dose (LD_{50}) and sublethal dose (LD_{10} and LD_{30}) at 24 and 48 h after exposure were calculated by Probit analysis using the Statplus program (version 2017, Analyst company, Canada).

Three extracts that showed the best control efficiency were chosen to make compound mixtures and subsequent analysis of the active ingredient compounds.

Isolation method

Major components of the fruits of *P. retrofractum* extract were isolated by preparative thin layer chromatography (PTLC) with 30% ethyl acetate (EtOAc) in hexane to yield a major compound identified as piperine (15.6%), whereas (2*E*,4*E*,14*Z*)-*N*-isobutylicosa-2,4,14-trienamide (6.2%) was obtained by PTLC using 10% EtOAc in hexane followed by 15% EtOAc in hexane. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 MHz AVANCE III HD spectrometer operating at 400 MHz (1H) and 100 MHz (13C). The high-resolution mass spectra (HRMS) were recorded on a MAXIS (Bruker).

Piperine

Pale yellow solid; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.39 (ddd, $J=14.7$, 8.4, 1.8 Hz, 1 H), 6.96 (d, $J=1.6$ Hz, 1 H), 6.87 (dd, $J=8.0$, 1.6 Hz, 1 H), 6.78–6.70 (m, 3 H), 6.42 (d, $J=14.7$ Hz, 1 H), 5.94 (d, $J=4.7$ Hz, 2H), 3.56 (s, 4 H), 1.74–1.46 (m, 6 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 165.59, 148.34, 148.26, 142.64, 138.38, 131.12, 125.51, 122.65, 120.20, 108.64, 105.82, 101.42, 47.07, 43.38, 26.87, 25.80, 24.81. HRMS (ESI) Calcd for $C_{17}H_{19}NNaO_3$ 308.1263 ($[M+Na]^+$), Found 308.1278.

(2E,4E,14Z)-N-isobutylicosa-2,4,14-trienamide

Pale yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.18 (dd, $J=15.0$, 9.9 Hz, 1H), 6.16–5.99 (m, 2H), 5.77 (d, $J=15.1$ Hz, 1H), 5.72 (s, 1H), 5.36–5.31 (m, 2H), 3.14 (dd, $J=12.3$, 5.8 Hz, 2H), 2.12 (dd, $J=13.7$, 7.1 Hz, 2H), 2.05–1.97 (m, 4H), 1.85–1.73 (m, 1H), 1.62 (dt, $J=15.2$, 7.8 Hz, 1H), 1.43–1.37 (m, 2H), 1.29 (ddd, $J=19.8$, 9.3, 4.0 Hz, 16H), 0.93–0.84 (m, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.72, 143.43, 141.55, 129.98, 128.32, 121.76, 47.10, 32.08, 31.89, 29.90–29.19, 28.93, 28.72, 27.30, 27.02, 22.45, 20.24, 14.11. HRMS (ESI) Calcd for $C_{24}H_{43}NNaO$ 384.3242 ($[M+Na]^+$), Found 384.3235.

For the ethanolic extract of the rhizomes of *A. calamus*, the major spot on TLC was isolated by PTLC using 25% EtOAc in hexane to obtain fraction 1 (17.1%). For structural analysis using ^{1}H NMR, this fraction was identified as a mixture of beta-asarone and alpha-asarone (ratio 4.38/1) and confirmed by authentic compounds purchased from Sigma-Aldrich.

Asarone mixture

Colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$): beta-asarone: δ 6.84 (s, 1H), 6.53, (s, 1H), 6.48 (m, 1H), 5.77 (dq, 1H, $J=11.5$, 7.0 Hz), 3.90 (s, 3H), 3.84 (s, 3H), 3.81 (s, 3H), 1.84 (dd, 3H, $J=6.6$, 1.8 Hz); alpha-asarone: δ 6.94 (s, 1H), 6.65 (dq, 1H, $J=15.8$, 1.7 Hz), 6.49 (s, 1H), 6.09 (dq, 1H, $J=16.0$, 6.6 Hz), 3.88 (s, 3H), 3.85 (s, 3H), 3.82 (s, 3H), 1.88 (dd, 3H, $J=6.6$, 1.8 Hz).

Contact toxicity bioassay for pure compounds

Similar to the crude extract, a contact toxicity bioassay was performed with second instar larvae of *S. litura*. Each purified compound was evaluated individually to determine their efficacy levels upon 2-μl topical application to the thorax region with various concentrations (2–100 μg/larva) using acetone as a carrier at concentration. Thirty insects at each concentration were used with five biological replicates. After treatment, larvae were maintained in the insect-rearing room and allowed to feed on an artificial diet. Mortality was recorded at 24 h post-treatment. The median lethal dose (LD_{50}) at 24 h after exposure was calculated by Probit analysis using the Statplus program (version 2017, Analyst Company, Canada).

Mixture concentration preparation methods

The compound mixtures of plants were prepared and modified from Hummelbrunner and Isman [13] by choosing the dose at LD_{30} or LD_{10} values of *S. litura* after contact toxicity analysis.

The LD_{10} and LD_{30} values of two extracts that showed the best control efficiency were chosen to make compound mixtures. Each crude extract was prepared to a specific concentration by dissolving in acetone. Then, 1 ml of each crude extract was mixed at a ratio of 1:1 v/v. The mixture was then used to analyze the contact toxicity and antifeedant efficiency.

Contact toxicity assay for compound mixtures

As described above, each mixture was treated with *S. litura* larvae by topical application. A minimum of 30 insects/combination was used for each experiment, and five replicates were performed. After 24 h, mortality was recorded. Actual mortalities were compared with expected mortalities based on the formula described as follows:

$$E = O_a + O_b(1 - O_a),$$

where E is the expected mortality and O_a and O_b are the observed mortalities of extracts at the given concentration. The effects of mixtures were designated

antagonistic, additive, or synergistic by analysis using χ^2 comparisons from the following formula:

$$\chi^2 = \left((O_m - E)^2/E\right),$$

where O_m is the observed mortality from the binary mixture and E is the expected mortality. In addition, χ^2 with $df = 1$ and $\alpha = 0.05$ is 3.84. A pair with χ^2 values > 3.84 and having higher than expected mortality was considered to be synergistic (negative = antagonist effect), with χ^2 values < 3.84 representing additive effects. An observed mortality less than expected suggested an antagonistic effect of the mixtures. The mixtures that showed a synergistic effect were used for antifeedant and enzyme assays.

Antifeedant bioassay for compound mixtures

The no-choice bioassay investigated the antifeedant effect. Each binary mixture was applied to kale leaf discs (4 cm^2) using a micropipette with 2 µl on each side [22] and allowed to air dry at room temperature before releasing early third instar larva onto the discs. Each larva that was starved for 4 h was placed in a Petri dish with one treated leaf disc and allowed to feed. Each treatment used 30 larvae with three replicates. The uneaten area of the leaf disc was measured using a digitizing leaf area meter after 3 h of feeding. The percent feeding inhibition was calculated by using the formula from [23], $(C-T)/(C+T) \times 100$, where C is the consumption of the control leaf and T is the treated leaf cut.

Enzyme assays

Enzyme extraction method

Enzyme assays were performed in an in vivo experiment. The combined mixtures were tested with the second instar of *S. litura* larvae to optimize its effect on detoxification enzyme activities. Acetone was used as a control group. After 24 h, the surviving larvae were used for enzyme extraction to determine the activities of esterase and glutathione-*S*-transferase. The extraction method was modified from Feyereisen [24], and surviving larvae were placed in a microtube and kept on ice. Then, larvae were ground with homogenized buffer (0.1 M potassium phosphate buffer mixed with 1 mM EDTA at pH 7.2). Homogenates were centrifuged at 4 °C and 12,000 rpm for 15 min. The supernatants were transferred to new tubes and kept on ice immediately to study the different enzyme activities.

Esterase activity (EST)

The esterase activity was determined by the method of Bullangpoti et al. [25] with modifications. Enzyme solution (40 µl) was mixed with *p*-nitrophenylacetate (pNPA) (10 mM in DMSO) and potassium phosphate buffer (50 mM, pH 7.4). Enzyme activity was measured at 410 nm and 37 °C for 90 s in a 96-well plate in a microplate reader using the kinetic mode. EST activity was determined using the extinction coefficient of 176.4705 for pNPA.

Glutathione-S-transferase activity (GST)

The glutathione-*S*-transferase method was modified from Oppenoorth et al. [26]. The mixtures containing 50 mM phosphate buffer (pH 7.2) were mixed with glutathione solution, supernatant, and 1-chloro-2,4′-dinitrobenzene (CDNB). Then, the activity of the mixtures was measured at a wavelength of 340 nm using a microplate reader. The GST activity was determined from the extinction coefficient of 0.000137 for CDNB. Three biological replicates per treatment were estimated.

Results

Extract yields

Alpinia galanga, *P. nigrum* and *P. retrofractum* extracts obtained were dark brown gum, whereas *S. trilobata* and *C. longa* extracts were dark green gum and orange gum, respectively. *A. calamus* extract was a yellow viscous semisolid. The percent yields were calculated by comparing the mass of crude extracts to the amount of fresh materials. The highest yield was obtained from ethanolic crude extraction from *C. longa* (Table 1).

Preliminary toxicity results of crude extracts

To determine the most effective extracts in this study, six ethanolic extracts were applied topically to second instar *S. litura* to assess toxicity. The median lethal dose values of each ethanolic extract are shown in Table 2. Among all extracts, *P. retrofractum* was the most effective extract against *S. litura*, followed by *A. calamus* and *P. nigrum*; whereas, *A. galangal*, *S. trilobata*, and *C. longa* were less efficient (Table 2). *A. calamus* and *P. retrofractum* were chosen for binary compound mixtures using LD_{10} and LD_{30} values. The LD_{10} and LD_{30} values were 3.294 and 6.735 µg/larva, respectively, for *A. calamus* and 1.448 and 3.213 µg/larva, respectively, for *P. retrofractum*.

Preliminary toxicity results of purified compounds

Base on the high toxicity of crude extracts, *A. calamus*, and *P. retrofractum* were chosen for analysis of the main effective chemical constituents for quality assessment of the mixture product for potential future development for commercial use. The major components from

Table 1 The amount of ethanolic crude extracts derived from six plant species

Crude extracts	Weight (g)	Percentage yield of plant extracts (% w/w) (%)	Appearance
C. longa	90.76	9.076	Orange gum
A. galanga	29.39	2.939	Dark brown gum
S. trilobata	28.20	2.820	Dark green gum
P. nigrum	31.03	3.103	Dark brown gum
A. calamus	23.07	2.307	Yellow viscous semisolid
P. retrofractum	12.02	2.405	Dark brown gum

Table 2 Toxicity of different ethanolic plant extracts (μg/larva) against second instars of *S. litura*

Compounds	*C. longa*		*S. trilobata*		*A. galanga*	
Dose	24 h	48 h	24 h	48 h	24 h	48 h
LD_{50}	108.894	106.171	41.993	41.141	30.5	29.703
LD_{50} SE	12.104	11.77	3.466	3.39	3.472	3.366
Chi square	31.26	20.83	8.83	7.95	3.72	3.74
LD_{50} LCL	90.81	88.492	35.64	34.913	24.27	23.643
LD_{50} UCL	240.274	136.542	49.239	48.207	37.882	36.831
Slope ± SE	2.30 ± 0.31	2.25 ± 0.29	2.28 ± 0.23	2.29 ± 0.23	1.64 ± 0.17	1.65 ± 0.17
Compounds	***P. nigrum***		***A. calamus***		***P. retrofractum***	
Dose	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**
LD_{50}	27.91	25.805	11.044	9.78	5.575	5.522
LD_{50} SE	3.227	2.891	0.87	0.804	0.522	0.507
Chi square	3.04	2.84	4.62	5.75	0.53	0.45
LD_{50} LCL	22.37	20.752	9.474	8.319	4.597	4.569
LD_{50} UCL	35.042	32.169	12.9	11.479	6.631	6.546
Slope ± SE	1.41 ± 0.13	1.44 ± 0.13	2.44 ± 0.22	2.30 ± 0.21	2.19 ± 0.27	2.24 ± 0.27

LD_{50} lethal dosage that kills 50% of the exposed larvae, expressed in μg/larvae; *SE* standard error; *LCL* lower confidence limit; *UCL* upper confidence limit

P. retrofractum were piperine (15.6%) and (2*E*,4*E*,14*Z*)-*N*-isobutylicosa-2,4,14-trienamide (6.2%); whereas, the major component from *A. calamus* was asarone (17.1%, ratio of beta/alpha = 4.38/1) (Table 2). The structures of isolated compounds are presented in Fig. 1. From Table 3, the toxicity results of all isolated compounds showed that (2*E*,4*E*,14*E*)-*N*-isobutylicosa-2,4,14-trienamide was the active compound of *P. retrofractum* (LD_{50} = 1.66 μg/larva) and that alpha-asarone was the active compound of *A. calamus* (LD_{50} = 2.22 μg/larva).

Contact toxicity of compound mixtures

The 2 best crude extracts at LD_{10} and LD_{30} doses were mixed, resulting in 4 paired combinations to investigate the synergistic effect of binary mixtures. Each mixture was used to topically treat *S. litura*, and mortality was observed at 24 h. This binary bioassay revealed that the combination of *P. retrofractum* and *A. calamus* exhibited synergistic toxicity against *S. litura* (Table 4).

Antifeedant effects of binary mixtures

The antifeedant activity of the binary mixtures was performed using a no-choice assay. The results showed that the percentage of feeding inhibition of binary mixtures was 15.12–82.43% after 3 h of feeding. Among these combinations, the mixture of 3.213 μg/larvae *P. retrofractum* with 3.294 μg/*larvae A. calamus* was very efficient with 82.43% feeding inhibition compared with others (Table 5).

Effect of binary mixtures on enzyme activities

Based on antifeedant activity, we hypothesized that this binary mixture affected detoxification enzyme activity. The results revealed that GST activity as assessed by in vivo assays was induced after treatment with mixtures (Table 6). Moreover, significant increases between the control and treated groups ($p < 0.05$, $df = 7$) in vivo were noted for GST (1.488 μg/larvae *P.*

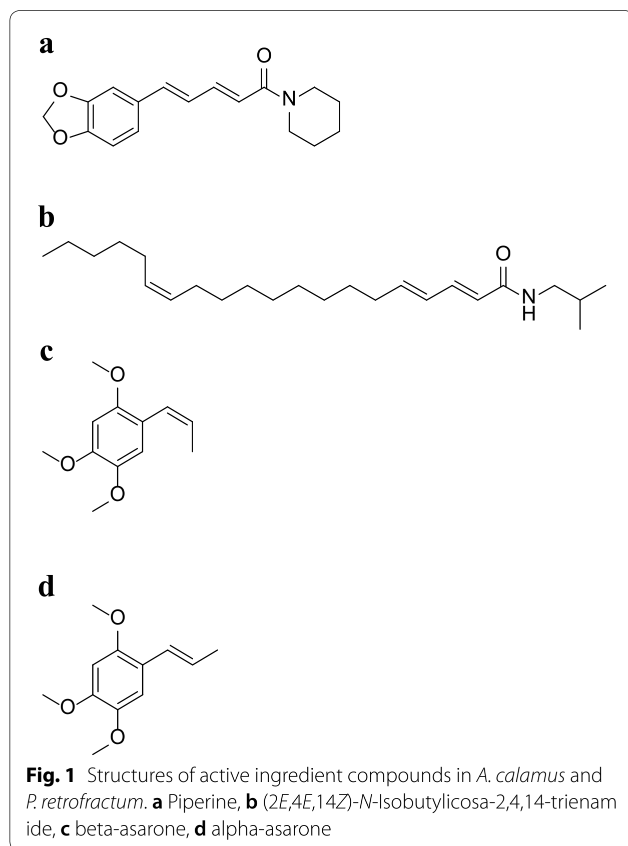

Fig. 1 Structures of active ingredient compounds in *A. calamus* and *P. retrofractum*. **a** Piperine, **b** (2*E*,4*E*,14*Z*)-*N*-Isobutylicosa-2,4,14-trienamide, **c** beta-asarone, **d** alpha-asarone

retrofractum + 6.735 μg/larvae *A. calamus* and 1.488 μg/larvae *P. retrofractum* + 3.294 μg/larvae *A. calamus*) (Table 6).

Discussion

Plant extracts as sources of bioactive and therapeutic purposes have been used for thousands of years [27]. Plant extracts and their derivatives have been evaluated for different pest control properties, e.g., their toxicity, repellent, antifeedant, fumigation, and effect on oviposition activities [28].

In this study, six plant extract compounds were extracted by ethanol, and the goal is that the farmer can use this knowledge to develop insecticides to control *S. litura* by themselves, potentially reducing the cost for pest control. All crude extracts evaluated via topical application against second instar *S. litura* larvae revealed that the ethanolic extract of *P. retrofractum* had the highest control efficiency, followed by *A. calamus* (Table 2). *P. retrofractum* and *A. calamus* are used as alternative medicines and supplements in primary health care worldwide [29, 30]. They are less toxic to nontarget organisms and mammals. A previous study from Wiwattanawanichakun et al. showed that *P. retrofractum* was moderately toxic for guppy fish compared with the known data available for synthetic pesticides [31]. Moreover, the LD_{50} values for oral doses of *A. calamus* extracts were greater than 5000 mg/kg body weight in Wistar rats [32] and 5070.59 mg/kg in mice [33].

Among the six plant extracts, *P. retrofractum* had the most potent extract with an LD_{50} value of 5.575 μg/larva. Similar results were reported by Chansang et al. [20]. Among aqueous extracts of nine medicinal plants, *P. retrofractum* showed the highest level of activity against *Cx. quinquefasciatus* and *Aedes aegypti* (L.) larvae with LD_{50}

Table 3 Toxicity of some active compounds (μg/larva) against second instars of *S. litura* at 24 h

	Plant source	LD_{50}	LD_{50} LCL	LD_{50} UCL	Slope ± SE	Chi square
Beta-asarone	*A. calamus*	6.24	4.62	8.66	1.44 ± 0.16	5.67
Alpha-asarone	*A. calamus*	2.22	1.74	2.61	2.94 ± 0.42	0.11
(2*E*,4*E*,14*Z*)-*N*-isobutylicosa-2,4,14-trienamide	*P. retrofractum*	1.66	1.34	1.95	2.19 ± 0.33	0.02
Piperine	*P. retrofractum*	81.56	36.71	181.27	2.10 ± 0.66	28.86

LD_{50} lethal dosage that kills 50% of the exposed larvae, expressed in μg/larvae; *SE* standard error; *LCL* lower confidence limit; *UCL* upper confidence limit

Table 4 Relative toxicity of binary mixtures against second instars of *S. litura* and measures of interactions

Crude A	Crude B	Compound alone		Binary Mixtures		χ^2	Effect
		Observed A	Observed B	Expected	Observed		
1.448 μg/larva *P. retrofractum*	3.294 μg/larva *A. calamus*	0.10	0.07	0.16	0.27	7.06	Synergistic
1.448 μg/larva *P. retrofractum*	6.735 μg/larva *A. calamus*	0.10	0.17	0.25	0.37	5.41	Synergistic
3.213 μg/larva *P. retrofractum*	3.294 μg/larva *A. calamus*	0.23	0.07	028	0.47	12.16	Synergistic
3.213 μg/larva *P. retrofractum*	6.735 μg/larva *A. calamus*	0.23	0.17	0.36	0.50	5.58	Synergistic

Table 5 The antifeedant activity induced by binary mixtures (1:1, v/v) against third instar *S. litura* larvae

Plant extract	Ratio	Antifeeding percentage
1.448 µg/larva *P. retrofractum* + 3.294 µg/larva *A. calamus*	LD_{10}:LD_{10}	15.12 ± 0.026
1.448 µg/larva *P. retrofractum* + 6.735 µg/larva *A. calamus*	LD_{10}:LD_{30}	42.69 ± 0.033
3.213 µg/larva *P. retrofractum* + 3.294 µg/larva *A. calamus*	LD_{30}:LD_{10}	82.43 ± 0.033
3.213 µg/larva *P. retrofractum* + 6.735 µg/larva *A. calamus*	LD_{30}:LD_{30}	63.22 ± 0.023

Table 6 Effect of plant-based binary mixtures on detoxification enzyme activities of second instar *S. litura* larvae

Mixture	Esterase[a]	Glutathione-*S*-transferase[b]
Control	0.64 ± 0.03a	1.61 ± 0.07a
1.448 µg/larva *P. retrofractum*	0.68 ± 0.06a	2.27 ± 0.13a
3.213 µg/larva *P. retrofractum*	0.69 ± 0.03a	1.64 ± 0.02a
3.294 µg/larva *A. calamus*	0.73 ± 0.08a	2.05 ± 0.03a
6.735 µg/larva *A. calamus*	0.72 ± 0.03a	1.674 ± 0.02a
1.448 µg/larva *P. retrofractum* + 3.294 µg/larva *A. calamus*	0.78 ± 0.04a	1.84 ± 0.02b
1.448 µg/larva *P. retrofractum* + 6.735 µg/larva *A. calamus*	0.76 ± 0.07a	1.9 ± 0.07b
3.213 µg/larva *P. retrofractum* + 3.294 µg/larva *A. calamus*	0.80 + 0.02a	1.79 ± 0.04b
3.213 µg/larva *P. retrofractum* + 6.735 µg/larva *A. calamus*	0.79 + 0.17a	1.7 + 0.06b

Means within a column followed by the same letter are not significantly different (ANOVA)

[a] Carboxylesterase activity ± SE (nM *p*-nitrophenol/min/mg protein)

[b] Glutathione-*S*-transferase activity ± SE (CDNB conjugated product/mg protein/min)

values of 135 and 79 µg/g, respectively. *P. retrofractum* exhibits insecticidal activity against many insect pests. It caused 100% mortality at a concentration of 0.5% against second instar *Crocidolomia pavonana* larvae [34]. This extract also exhibited a high knockdown effect on several test insects, including *P. xylostella*, *C. pavonana*, *Cx. quinquefasciatus*, *Ae. aegypti*, and *Coptotermes gestroi* [20, 35].

Another Piperaceae plant in this research is *P. nigrum*, which is also among the top three for *S. litura* control. Kumar et al. [36] revealed that the ethanolic extracts of black and white *P. nigrum* were 30–40% less toxic than the extracts of *Piper longum* against *A. aegypti* larvae. Additionally, a hexane extract showed a toxic effect against second instar larvae of *S. litura* with an LD_{50} of 1824 µg/g insect [37].

Some reports described that the active compound extracted from the fruit part of *P. retrofractum* and *P. nigrum* is piperine [38, 39]. This compound also showed insecticidal activity against larvae of plant insects and antimicrobial activity [38]. However, in our results, piperine does not provide efficient control of *S. litura*. Our study assessed another new active ingredient from this piperaceae plant crude extract called (2*E*,4*E*,14*Z*)-*N*-isobutylicosa-2,4,14-trienamide.

The other crude extract that showed the highest *S. litura* control in our results is *A. calamus*. Many papers have been published on the biological activities of *A. calamus*. A total of 0.4% of ethanolic extracts yielded 63.3% mortality against third instar larvae of *P. xylostella* based on the leaf-dipping method [40]. Phongpaichit et al. [41] revealed that the crude methanol extract exhibited high antimicrobial activity on various microorganisms and fungi. The larvicidal activity of *A. calamus* is due to the presence of the primary chemical compound beta-asarone [17].

Schmidt and Streloke [42] investigated the chemical composition of *A. calamus* rhizome using beta-asarone as the major compound for control *Prostephanus truncatus* (Horn). Other chemicals investigated in *A. calamus* include ethyl isoeugenol, 3.9-decadien-*ol*-1,3-methyl-6-(1-methylethenyl), 4-pentyl-1-(4propylcyclohexyl)1cyclohexene, and alpha-asarone, which also has toxicity to insects, such as *S. litura* and *Liposcelis bostrychophila*, and other bioactivities [43–45].

Furthermore, synergistic effects of complex mixtures are thought to be important in plant defenses against herbivory. Plants usually present defenses based on a group of compounds and not individual compounds [13]. Although the highest mortality was noted with the *P. retrofractum* extract compared with other extracts at the

same dose in our study, the combination of each extract in a binary assay produced stronger toxicity.

In this study, *P. retrofractum* synergized the toxicity of *A. calamus* at all doses. Among all combinations, the mixture of *P. retrofractum* + *A. calamus* (LD_{30}:LD_{10}) could be the chosen mixture for controlling this insect. Both combinations showed a synergistic effect and also exhibited higher antifeedant activity at 82.43% compared with others (Tables 4, 5).

Dadang et al. [46] reported the robust efficacy of a mixture of *P. retrofractum* with *Annona squamosa* and *Aglaia odorata*, which produced 100% and 94% mortality, respectively, in *Crocidolomia pavonana* after a 48-h treatment with a 0.05% extract mixture. The extract mixture of *A. odorata* and *A. squamosa* yielded a synergistic combination with multiple actions, such as feeding inhibition and insecticidal activity [46].

Insects have well-developed defense mechanisms against insecticides and natural pathogens that involve various enzyme systems. It is well known that herbivorous insects use detoxification enzymes, including EST, GST, and cytochrome P450 monooxygenases, to metabolize toxic chemicals and secondary plant metabolites [47]. However, these enzymes are also induced by xenobiotics as one of the mechanisms responsible for the development of resistance in insects [48]. The enzyme activities from in vivo treatment are presented in Table 5. GST activities in treated insects that survived after 24 h of exposure revealed a significant increase in GST in the *P. retrofractum* + *A. calamus* mixture at all combinations compared with the control group (Table 6). Conversely, EST activity showed no significant difference between groups ($p \geq 0.05$, $df = 7$) (Table 6). Kaur et al. [49] reported that the induction of detoxification enzyme activities depended on both the duration of treatment and concentrations [50]. Specifically, a prolonged treatment and high dose showed a higher increase in enzyme activity. Therefore, the ratio and concentration of the mixed compounds are essential given that a suitable concentration could possibly affect detoxification enzymes.

An induction of detoxification enzyme activity has been reported by Zhou et al. [51], demonstrating that extracts from *Illicium verum* fruit induced EST activity in *Myzus persicae* and also increased EST activity in the gypsy moth *Lymantria dispar* after feeding on a diet of aspen leaves supplemented with phenolic glycosides [52]. A similar result was found in *S. litura* after they fed on *Melia toosendan* extract, demonstrating that midgut esterase activities were significantly increased after 24 and 48 h of feeding and decreased after 72 h [53].

Conclusion

The results demonstrated that ethanolic crude extracts of *A. calamus* L., *A. galangal*, *C. longa* L., *P. nigrum*, *P. retrofractum*, and *S. trilobata* caused toxicity in *S. litura*. Additionally, binary mixtures of *P. retrofractum* and *A. calamus* showed synergistic effects, suggesting that these mixtures could serve as an acute toxicant or antifeedant. In particular, the mixture of *P. retrofractum* + *A. calamus* (LD_{30}:LD_{10}) at this combination exhibited the highest antifeedant activity at 82.43%, showing synergistic contact toxicity effects and no significant differences in both EST and GST activities compared with controls. Therefore, the combination of these two compounds may constitute a useful alternative approach for the development of a binary mixture formulation to control lepidopteran pests for use in an IPM system.

Abbreviations

PTLC: preparative thin layer chromatography; IPM: integrated pest management; EtOAc: ethyl acetate; GST: glutathione-*S*-transferase; EST: esterase; pNPA: *p*-nitrophenylacetate; CDNB: 1-chloro-2,4′-dinitrobenzene; ANOVA: analysis of variance.

Authors' contributions

VB and WP designed the experiment. TY, AP and AR performed the experiments. VB and WP wrote and reviewed the paper. VB and WP checked all the details. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data are presented in Tables 1, 2, 3, 4, 5, 6.

Consent for publication

This research has been confirmed for publication in the journal.

Ethics approval and consent to participate

All experimental procedures in this research were performed with the approval of an appropriate animal Ethics Committee of Kasetsart University, Thailand, under reference number OACKU01059.

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