Chemical and Biological Technologies in Agriculture

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Synergistic interaction of thymol with *Piper ribesioides* (Piperales: Piperaceae) extracts and isolated active compounds for enhanced insecticidal activity against *Spodoptera exigua* (Lepidoptera: Noctuidae)

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Abstract

Background: Plant secondary metabolites or mixtures in extracts or essential oils are well known to enhance the activity in binary mixtures. The present study is the first to report that thymol synergistically or additively enhances the activity of *P. ribesioides* extracts and isolated compounds against *S. exigua* larvae at sublethal doses.

Results: Thymol was synergistic when are mixed with hexane extract; however, if the hexane extract level was higher (LD_{30}) than the thymol level (LD_{10}), the reaction was antagonistic. CH_2Cl_2 extract and thymol were more toxic than the extract or thymol alone, and EtOAc extract was synergized by thymol if the components were combined at similar levels (1:1 thymol:EtOAc extract at the LD_{10} or LD_{30}). MeOH extract individually had moderate insecticidal activity, but all combinations with thymol were synergistic as binary mixtures. Isolated compounds, piperine, phenethyl cinnamamide and cinnamic acid represented synergistic, additive, and antagonistic action after combining with thymol (1:1 at the LD_{10} or LD_{30}). Detoxification enzymes after exposure of insects to treatments showed isolated compounds + thymol could inhibit CE, GST and AChE reaction of *S. exigua* exceptional being piperine + thymol, which induced detoxification enzyme activity.

Conclusion: The synergistic activity was extract- and dose-specific. The impact on detoxification enzymes was variable and dependent on the composition of the extract and the doses of extract and thymol used in a binary mixture. In this metabolic model, the major insect compound in an extract may become detoxified, whereas a minor compound will act unimpeded, showing a lower LD_{50} than acting alone. This model suggests that thymol synergizes with extract components differently, which could depend on the specific metabolites in the extract and the dose applied. Such studies will help design effective insecticides based on natural plant mixtures and a synergistic compound.

Keywords: Thymol, *Piper ribesioides*, *Spodoptera exigua*, Synergistic, Acute toxicity, Detoxification enzyme, Binary mixtures

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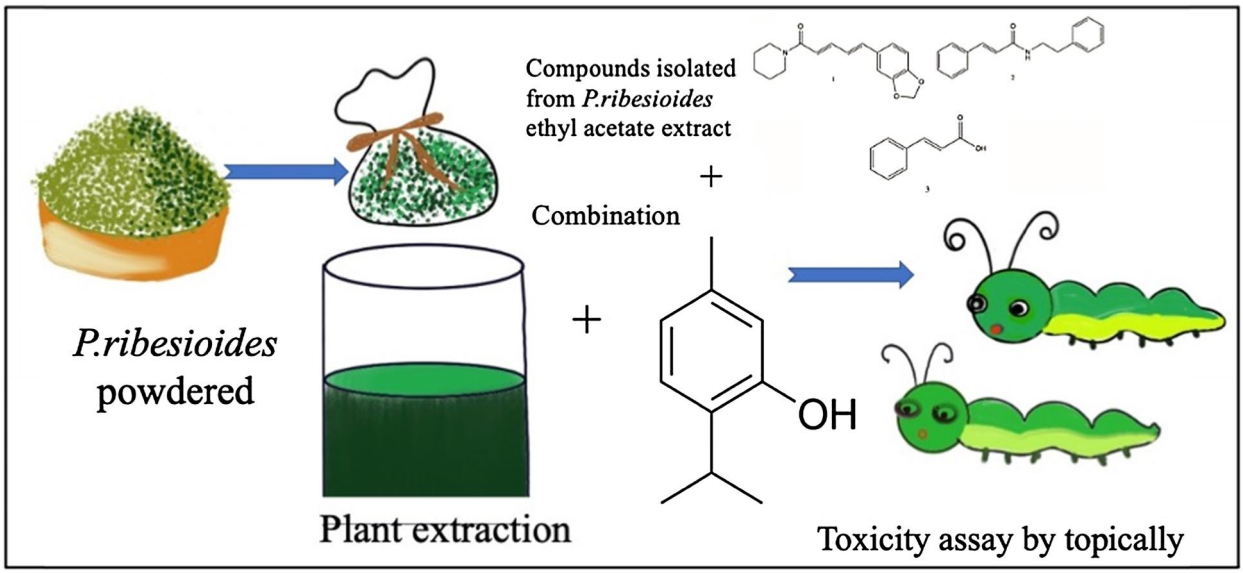
Graphical abstract



Introduction

Conventional insecticides such as chlorantraniliprole, tetraniliprole, methoxyfenozide, indoxacarb, chlorfenapyr, emamectin benzoate and β-cypermethrin are used to prevent herbivory by *Spodoptera exigua*, which is a polyphagous insect infesting cereals, legumes, cotton, tobacco and cannabis that causes severe economic losses worldwide [1]. The use of such hazardous chemicals has resulted in resistance to these insecticides, environmental contamination, and mortality of beneficial insects [1, 2]. In contrast, botanical insecticides such as pyrethrum, neem, rotenone, nicotine and plant essential oils protect crops and stored products to avoid such hazards of chemical insecticides [3–6]. Several recent studies have demonstrated the efficacy of essential oil compounds against lepidopterans [7, 8]. Thymol is a well-known monoterpenoid found in the essential oil of *Thymus vulgaris L.*, commonly known as thyme. It also occurs in several other plant genera, such as *Ocimum*, *Origanum*, *Carum*, *Satureja*, and *Oliveria.* It has been used in the medical, dental, veterinary, food, and agrochemical industries [9]. Its agricultural applications as an insecticide, acaricide, and repellent have also been reported [10].

Commonly, botanical insecticides, including volatile oils, are considered safer than conventional insecticides due to their biodegradability; variable modes of action; and low toxicity to mammals, birds and fish [11]. While the biodegradable nature of botanical pesticides is advantageous for the environment and human health, the short shelf lives of these pesticides make developing appropriate biopesticide formulations difficult. One of the approaches in the recent past has been to use binary mixtures with synergistic activity against insects that can solve both problems, increasing the shelf life of a formulation while minimizing the environmental impact [5, 12].

Piper ribesioides is a Piperaceae plant that is widely distributed in tropical and subtropical regions and is now known to have insecticidal and antifeedant properties [8]. Similarly, thymol is obtained from *Thymus vulgaris* (Labiatae) and some other aromatic plants and is reported to have insecticidal, acaricidal and antifeedant activities [13, 14]. One objective of this research was to determine whether *S. exigua* larval control efficiency could be increased while reducing synthetic pesticide usage by using a mixture of compounds from plant extracts such as *Piper ribesioides* extracts and thymol.

Our previous research found that both thymol and *P. ribesioides* extract [15–17] has insecticidal activity. However, farmers always prefers have quick pest control efficiency. Thus, the increase strategies of botanical extract need to be developed. Our present study is the first to establish the synergistic effects of such binary mixtures based on a combination of *P. ribesioides* extracts and the botanical insecticide thymol. The main hypothesis goal is to increase control efficiency of this mixture product compare to alone compound.

In addition, as the control of these pests requires the massive use of insecticides, they have developed resistance to all chemical families. This *Spodoptera* species present in the Arthropod Pesticide Resistance Database are in the top 15 most resistant arthropods on the planet: *S. litura*, *S. frugiperda* and *S. exigua* (Hübner) [18, 19]. Since knowledge of the stability of resistance is a crucial aspect when recommending rotation of insecticides with different modes of action [20], here, we examined beet armyworm stability of resistance to our mixed compounds. Our other purposes were to determine the impact of such mixtures on detoxification and neural enzyme activities and to investigate whether such combinations would inhibit these enzymes and prevent the possibility of developing resistance.

Materials and methods

Plant

P. ribesioides plant material was collected from the Nan Province of Northern Thailand (latitude: 19.490850 and longitude: 100.90199). The botanists of the Forest Herbarium identified the plant species, which belong to the Department of National Park, Wildlife and Plant Conservation, Bangkok, Thailand (specimen voucher number BK068730).

Plant extraction

Twigs (4 kg) of *P. ribesioides* were dried under shade and then powdered using a commercial blender. The obtained powder (1 kg each) was extracted separately with four organic solvents, hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and methanol (MeOH), for 7 days each using a soaking procedure [8]. Each extract was filtered through a suction filter (Whatman no. 0) with a vacuum pump and then concentrated using a rotary evaporator (BUCHI R-215). The quantitative yield of each extract was recorded, and the extract was then stored at 4 °C until further use in biological assays.

Isolation of compounds

The crude EtOAc (16.90 g) was separated by Si-gel CC (SiO_2, 600 g, CH_2Cl_2–hexane and MeOH–CH_2Cl_2 gradient elutions) to give fractions A_1 to A_5. Fraction A_3 (3.20 g) produced fractions B_1 to B_4 after Si-gel CC (10% acetone–hexane isocratic). B_1 gave colorless needles after recrystallization from acetone–hexane and was identified as piperine (1) (101.10 mg). Fraction A_4 (2.13 g) was subjected to Si-gel CC (30% EtOAc–hexane isocratic as eluent) to provide 4 fractions (C_1–C_4). Fraction C_2 (1.21 g) accorded fractions D_1 to D_3 after CC on Si-gel (10% EtOAc–hexane isocratic as eluent). An addition 7.32 g of the fraction A_5 was fractionated by Si-gel CC using isocratic elution mixture of 30% CH_2Cl_2 in hexane as eluent to obtain E_1 to E_4 fractions. Subfraction E_4 (3.40 g) was chromatographed on a Sephadex LH-20 column isocratic eluted with MeOH to give 5 fractions (F_1–F_4). Fraction F_4 was recrystallized from MeOH–CH_2Cl_2 to provide phenethylcinnamamide (2) (195.90 mg). Fraction F_3 (108.10 mg) provided cinnamic acid (3) (89.20 mg) after recrystallization from MeOH.

Melting points (uncorrected) were recorded in °C and were determined on a digital Electrothermal IA 9000 series apparatus for all the compounds. The FT-IR and UV spectra were recorded on a spectrum GX-FT-IR system (Perkin Elmer) and JASCO V-530 spectrophotometers, respectively. NMR spectra were recorded in deuterated chloroform and deuterated methanol on Bruker Ascend TM 400 spectrometer using TMS. HRMS-TOF on Micro mass model VQ-Tof2. Low-resolution EI mass spectra were recorded on a Thermo Finnigan Polaris Q mass spectrometer at 70 eV (probe). Silica gel 60 (Merck, 70e230 mesh) and silica gel plates (Merck, Silica gel 60 PF254) were used for CC and preparative TLC, respectively.

Insects

S. exigua larval colonies obtained from the National Center for Genetic Engineering and Biotechnology, Bangkok, were transferred to an insect rearing room and maintained on an artificial diet. The protocol was approved by the committee overseeing animal care and use in scientific research under approval no. ACKU64-SCI-019. The diet comprised commercial green bean powder (110 g) (Raithip®), agar (11 g), ascorbic acid (1.5 g), sorbic acid (1 g), methylparaben (2 g), yeast (8.5 g), mixed vitamins (17 mL), formaldehyde (1 mL), and distilled water (680 mL) and was used with a procedure modified from that of Nobsathian et al. [16]. Net cages (50 × 50 cm, 50 cm high) were the arenas for adult emergence from developed pupae. Cotton swabs dipped in 10% sugar solutions placed in a small plastic dish (5 cm in diameter, 15 cm high) were used as food for the adults in net cages. The cages were also lined on two sides with wax sheets for the oviposition of moths. Egg colonies were collected daily and sterilized by dipping in 10% formaldehyde for 10 s to prevent infections. All stages of *S. exigua* were maintained under laboratory conditions of 27 ± 2 °C, 60–70% relative humidity and a 14:10 h (light:dark) photoperiod.

Larvicidal bioassay

The thymol used in the current study was obtained commercially (99% purity) from Sigma-Aldrich (Bangkok). Thymol and *P. ribesioides* extracts were applied topically to 2nd instar *S. exigua* in the thorax region (2 µL/larva). For each treatment, 30 larvae were used in 3 replicates

($n=90$). Treated larvae were placed in sealed plastic petri dishes and fed an artificial diet. The petri dishes were covered with black paper to prevent phototaxis and maintained under the laboratory conditions described above. Acute sublethal toxicity due to each treatment was assessed after 24 h, and the mortalities were used to calculate the LD_{10} and LD_{30} using a probit analysis program (STATPLUS, version 2019).

Synergistic assay

For the preparation of binary mixtures, the LD_{10} and LD_{30} were used as the sublethal doses of various treatments. Combinations of thymol and extracts were made by mixing thymol with individual extracts prepared in hexane, CH_2Cl_2, EtOAc, and MeOH in a 1:1 ratio at the specified sublethal doses. Thus, four binary mixtures with thymol (one for each extract) were assayed for acute toxicity as described above for the individual extracts. After treatment, the mortalities due to the mixtures used were compared to the expected mortalities based on the formula [21] described below:

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

where E is the expected mortality and O_a and O_b are the observed mortalities of individual treatments at the given concentration. The efficacies of the binary mixtures were determined to be antagonistic, additive, or synergistic by χ^2 comparisons:

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

where O_m is the observed mortality due to the binary mixtures, E is the expected mortality, and χ^2 with $df=1$ and $\alpha=0.05$ is 3.84. χ^2 values > 3.84 and greater-than-expected mortality were considered to indicate synergistic effects, while χ^2 values < 3.84 indicated additive effects. Similarly, lower-than-expected observed mortality rates were taken to indicate an antagonistic effect of a mixture.

Detoxification enzymes

Detoxification enzyme reactions were determined only for binary mixtures that were synergistic in bioassays.

Enzyme source

The enzyme was prepared from *S. exigua* larvae treated with synergistic mixtures using a modified procedure from Kumrungsee et al. [7] and Nobsathian et al. [8]. Surviving 2nd instars of *S. exigua* were taken for this study (5 larvae/replicate) and homogenized by using a pestle in a microtube in 100 mM phosphate buffer (pH 7.2) and Triton *X*-100 (0.5%). All homogenates were centrifuged for 15 min at 12,000 rpm at 4 °C. The supernatants were collected carefully and stored at −20 °C for further use as enzyme sources.

Carboxylesterase (CE)

The reaction of CE was analyzed by a procedure modified from those of Kumrungsee et al. [7] and Nobsathian et al. [8]. The enzyme (50 µL) from the enzyme source was preincubated at 30 °C for 30 min. The substrate was prepared, which contained 10 mM paranitrophenylacetate (pNPA) (50 µL) and 50 mM phosphate buffer (pH 7.2, 2.9 mL). The enzyme and substrate solutions were homogenized and transferred to 96-well microreader plates to measure the enzyme activity (at 400 nm, 25 °C, 3 min) in the kinetic mode under an extinction coefficient 176.4705 for pNPA. Three biological replicates per treatment were analyzed.

Glutathione-s-transferase (GST)

The procedures of Kumrungsee et al. [7] and Nobsathian et al. [8] were again followed to measure GST activity. Enzyme solution (20 µL) was mixed thoroughly with a substrate mixture of 0.1 phosphate buffer (0.1 M, pH 7.2, 1150 µL) and 1-chloro-2,4-dinitrobenzene (CDNB) (150 mM, 10 µL). The glutathione-s-transferase reaction was measured by using a 96-well microplate reader at 25 °C for 3 min, and the optical density was measured at 340 nm. The CDNB level was determined from the extinction coefficient of 0.000137 for the GST reaction. Three replicates per treatment were analyzed.

Acetylcholinesterase (AChE)

A procedure modified from that of Ellman [22] was used to measure the activity of AChE. The enzyme (50 µL) was preincubated for 30 min at 30 °C. The substrate solutions contained phosphate buffer (pH 7.2, 100 mM, 50 µL), 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) in 0.1 M ethylenediamine tetraacetic acid (EDTA) and 100 mM acetylthiocholine iodide (AsCH). The enzyme and substrate solutions were mixed thoroughly. The AChE reaction was measured at 412 nm with an extinction coefficient of $1.36 \times 104\ M^{-1}\ cm^{-1}$. Three replicates per treatment were evaluated.

Results

Plant extracts

The extracts obtained from the twigs of *P. ribesioides* varied quantitatively. The maximum yield recorded was for the hexane fraction, followed by the EtOAC, and MeOH fractions (Table 1).

Isolated compounds

The active compounds were isolated from ethyl acetate crude extracts of *P. ribesioides*. All pure compounds

Table 1 Extracted amount of *P. ribesioides* twigs in different solvents

Solvents	Characteristics	Yield (%)[a]
Hexane	Light yellow sticky oil	0.3732
CH_2Cl_2	Light gray sticky extract	0.2265
EtOAc	Brown sticky extract	0.2797
MeOH	Dark brown sticky extract	0.0432

[a] Yield (%) = *W*/Wt × 100, where W is the weight of dried extract and Wt is the weight of the *P. ribesioides* powder used for extraction

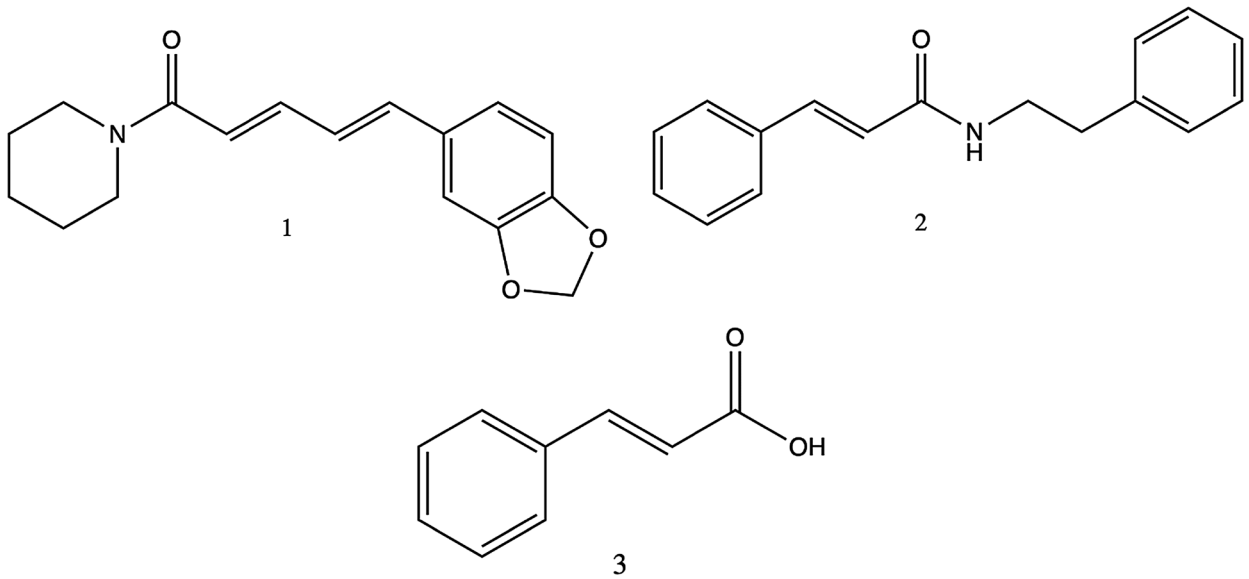


Fig. 1 Compounds isolated from *P. ribesioides* ethyl acetate extract. All compounds were purified and their structures were elucidated based on spectroscopic data as described in the text

were verified by comparing their physical properties and spectroscopic data with those reported in the literature [23–25]. The compounds (Fig. 1) were isolated and identified by spectral analysis as follows:

Piperine (1) [23]: colorless needles; m.p. 130–131.3 °C; UV (chloroform) λ_{max} (log ε): 343 (3.21), 311 (2.34), 263 (4.11), 255 (2.56) nm; IR (KBr, cm^{-1}) υ_{max} = 3010, 2950, 1666, 1580, 1450, 1340, 1032.

Phenethylcinnamamide (2) [24]: colorless crystal; m.p. 125–126 °C; UV (EtOH) λ_{max} (log ε):228 (3.96), 333 (1.73).; IR (KBr, cm^{-1}) υ_{max} = 3300, 3100, 3060, 2960, 2960, 1670, 1620, 1570, 1460, 1350, 1230, 1000, 870, 770, 750, 730, cm^{-1}.

Cinnamic acid (3) [25]: white powder; m.p. 193.9–209.3 °C; UV (MeOH) λ_{max} (log)272 (2.85) nm.; IR (KBr, cm^{-1}) υ_{max} = 3448, 1711, 1638, 1577, 1551, 1450 cm^{-1}.

Toxicity at sublethal doses

Analysis of the toxicity of thymol at sublethal doses revealed an LD_{10} of 1.21 µg/larva and an LD_{30} of 9.54 µg/larva. The methanol extract was less active at the LD_{10} dose than the hexane, CH_2Cl_2, and EtOAc extracts, which were not significantly different, as was evident due to the overlap of the fiducial limits (Additional file 1: Data S1). However, to obtain 30% mortality with the CH_2Cl_2 and EtOAc extracts, the LD_{30} doses required were similar. In the case of hexane treatment, this dose was higher than that needed for the CH_2Cl_2 and EtOAc extracts (Additional file 1: Data S1). Overall, the MeOH extract was the least active for both sublethal applications.

When used as binary mixtures, the combinations of thymol with hexane extract at sublethal doses were generally synergistic; however, if the amount of the hexane extract was higher (LD_{30} level) than that of thymol (LD_{10} level), the interaction was antagonistic (Table 2). Although moderately toxic toward *S. exigua* by itself, the CH_2Cl_2 extract showed enhanced toxicity after the addition of thymol at both the LD_{10} and LD_{30} doses. The EtOAc extract, significantly more active than the other *P. ribesioides* extracts, showed enhanced activity if combined with thymol at a similar level (1:1 thymol:ethyl extract at the LD_{10} or LD_{30}). The effects were antagonistic or additive if the components were combined at different doses (Table 2). MeOH extract individually had moderate insecticidal activity, but binary mixtures of the

Table 2 Potential toxicity due to binary mixtures of thymol and *P. ribesioides* extracts against 2nd instar *S. exigua* after 24 h

Compound A	Compound B (*P. ribesiodes* extracts in respective solvents)	Sublethal dose (µg/larvae)	Laval mortality (%)				χ^2	Effect
			Pure compounds		Binary mixtures			
			Observed A	Observed B	Expected	Observed		
Thymol (LD_{10})	Hexane (LD_{10})	1.21 + 0.65	13.33	23.33	33.56	53.33	11.66	Synergistic
Thymol (LD_{10})	Hexane (LD_{30})	1.21 + 3.21	13.33	26.67	36.44	6.67	24.33	Antagonistic
Thymol (LD_{30})	Hexane (LD_{10})	9.54 + 0.65	17.00	13.00	27.79	46.67	12.82	Synergistic
Thymol (LD_{30})	Hexane (LD_{30})	9.54 + 3.21	17.00	27.00	39.41	93.33	73.78	Synergistic
Thymol (LD_{10})	CH_2Cl_2 (LD_{10})	1.21 + 0.32	13.33	16.67	27.78	16.67	4.44	Antagonistic
Thymol (LD_{10})	CH_2Cl_2 (LD_{30})	1.21 + 1.73	13.33	20.00	30.67	86.67	102.26	Synergistic
Thymol (LD_{30})	CH_2Cl_2 (LD_{10})	9.54 + 0.32	17.00	17.00	31.11	56.67	20.99	Synergistic
Thymol (LD_{30})	CH_2Cl_2 (LD_{30})	9.54 + 1.73	17.00	20.00	33.60	96.67	118.38	Synergistic
Thymol (LD_{10})	EtOAC (LD_{10})	1.21 + 0.57	13.33	23.33	33.56	46.67	5.12	Synergistic
Thymol (LD_{10})	EtOAC (LD_{30})	1.21 + 1.60	13.33	27.00	36.44	10.00	19.19	Antagonistic
Thymol (LD_{30})	EtOAC (LD_{10})	9.54 + 0.57	17.00	23.00	36.09	43.33	1.45	Additive
Thymol (LD_{30})	EtOAC (LD_{30})	9.54 + 1.60	17.00	27.00	39.41	90.00	64.94	Synergistic
Thymol (LD_{10})	MeOH (LD_{10})	1.21 + 2.52	13.33	6.67	18.80	70.00	139.44	Synergistic
Thymol (LD_{10})	MeOH (LD_{30})	1.21 + 39.74	13.33	10.00	21.70	53.33	46.11	Synergistic
Thymol (LD_{30})	MeOH (LD_{10})	9.54 + 2.52	17.00	7.00	22.81	80.00	143.39	Synergistic
Thymol (LD_{30})	MeOH (LD_{30})	9.54 + 39.94	17.00	10.00	25.30	96.67	201.31	Synergistic

MeOH extract with thymol at all levels showed increased toxicity.

The active compounds identified were piperine, phenethylcinnamamide and cinnamic acid. The LD_{50} calculated was in the range of 0.17 to 1.64 µg/larva and LD_{90} of 3.39 to 117.82 µg/larva 24 to 48 h post-treatment (Additional file 1: Data S2). The toxicity at sublethal doses (LD_{10} and LD_{30}) was 0.04 to 0.13 and 0.16 to 0.39, respectively (Additional file 1: Data S3). The piperine with thymol was synergistic when combined at LD_{10} and LD_{30} doses or vice versa. However, if the combination was at similar sublethal amounts, the activity was either additive or antagonistic (Table 3). In the case of phenethylcinnamamide and thymol, the synergistic action was seen when combined at LD_{30} and LD_{10} doses (1:1), respectively (Table 3). Similarly, thymol was synergistic at the LD_{10} level for cinnamic acid, independent of the cinnamic acid dose of LD_{10} or LD_{30}. If the thymol dose was increased to LD_{30} in the mixture, the activity changed to additive or antagonistic (Table 3).

Detoxification enzymes

The enzyme activity of the extracts with thymol was determined only for those that were synergistic in toxicity assays. The impacts of treatment with binary mixtures of thymol and *P. ribesioides* extracts on 2nd instar *S. exigua* carboxylesterase were variable. Thymol and hexane extract inhibited CE, while thymol + CH_2Cl_2 and thymol + EtOAc extracts induced the enzymatic reactions. Similarly, thymol + MeOH extract had both inhibitory and inducing actions dependent on the dose of thymol used in the mixture; i.e., if thymol was used at the LD_{10}, inhibition of the enzyme was observed, whereas if it was used at the LD_{30}, induction was recorded (Table 4). Similar significant inhibition and induction compared to those in the control group were observed for GST and AChE reactions after binary mixture treatment of *S. exigua* larvae (Table 4).

The reaction of the isolated compounds with thymol was determined, cinnamic acid (LD_{10}) and thymol represented the highest impacts of inhibited CE, while piperine (LD_{10}) + thymol (LD_{30}) induced the enzyme activities. Thymol also significantly inhibited/induced GST and AChE reactions (Table 5).

Discussion

The roles of phytochemicals in integrated pest management strategies for sustainable food safety and agricultural production are well documented [12, 26] because of the challenges we have faced in terms of the human and environmental hazards caused by conventional chemical pesticides via the food chain, water sources, pesticide resistance and impacts on natural enemies [27]. Therefore, the current study was an attempt to show that the toxicity of *P. ribesioides* extracts, which have been reported to have insecticidal effects on *S. exigua* larvae [7], can be further enhanced by the addition of the known natural insecticidal compound thymol from a

Table 3 Potential toxicity due to binary mixtures of thymol and isolated compound of *P. ribesioides* extracts against 2nd instar *S. exigua* after 24 h

Compound A	Compound B (*P. ribesiodes* extracts in respective solvents)	Sublethal dose (µg/larvae)	Laval mortality (%)				χ^2	Effect
			Pure compounds		Binary mixtures			
			Observed A	Observed B	Expected	Observed		
Piperine (LD_{10})	Thymol (LD_{10})	0.04 + 1.21	13.33	13.33	24.31	30.00	1.33	Additive
Piperine (LD_{10})	Thymol (LD_{30})	0.04 + 9.54	13.33	16.67	27.79	56.67	30.01	Synergistic
Piperine (LD_{30})	Thymol (LD_{10})	0.16 + 1.21	16.67	13.33	27.79	53.33	23.47	Synergistic
Piperine (LD_{30})	Thymol (LD_{30})	0.16 + 9.54	16.67	16.67	31.11	10.00	14.32	Antagonistic
Phenethylcinnamamide (LD_{10})	Thymol (LD_{10})	0.13 + 1.21	23.33	13.33	33.01	53.33	12.51	Synergistic
Phenethylcinnamamide (LD_{10})	Thymol (LD_{30})	0.13 + 9.54	23.33	16.67	36.09	46.67	3.10	Additive
Phenethylcinnamamide (LD_{30})	Thymol (LD_{10})	0.36 + 1.21	30.00	13.33	39.10	63.33	15.02	Synergistic
Phenethylcinnamamide (LD_{30})	Thymol (LD_{30})	0.36 + 9.54	30.00	16.67	41.90	33.33	1.75	Antagonistic
Cinnamic acid (LD_{10})	Thymol (LD_{10})	0.11 + 1.21	6.67	13.33	19.09	40.00	22.90	Synergistic
Cinnamic acid (LD_{10})	Thymol (LD_{30})	0.11 + 9.54	6.67	16.67	22.81	16.67	1.65	Additive
Cinnamic acid (LD_{30})	Thymol (LD_{10})	0.39 + 1.21	26.67	13.33	36.49	60.00	15.15	Synergistic
Cinnamic acid (LD_{30})	Thymol (LD_{30})	0.39 + 9.54	26.67	16.67	39.41	36.67	0.19	Antagonistic

plant source [10]. Such combinations work in two potential ways: first by exerting synergistic and additive effects and second by reducing the chance of resistance development due to the multicomponent nature of the product. Previous reports have suggested that combining two compounds, thymol and pulegone (0.22 + 20 µg/larva), to combat *Plutella xylostella* larvae enhances toxicity by 2- to 3-fold compared to that of thymol or pulegone alone [7]. Binary mixtures of piperine and β-asarone improve feeding deterrence in *Spodoptera litura* and *Mythimna separata*, while the individual compounds [28, 29] causes inhibition.

Combinations of extracts and essential oils have also been reported to be synergistic. For instance, several combinations of *Allium sativum* and *Citrus paradisi* extracts are synergistic and are most potent against adult mosquitoes when applied in 1:1 ratios [31]; furthermore, some combination formulations of *Cymbopogon citratus*, *Illecium verum*, and *Myristica fragrans* essential oils are strongly synergistic against *Musca domestica* and are more effective than cypermethrin [32]. Similarly, the insecticidal activity of rosemary oil appears to be a consequence of the synergistic interaction between 1,8-cineole and (±)-camphor, where (±)-camphor is considered a promising synergizing agent against the cabbage looper, *Trichoplusia ni* [30].

The objective of the present study was to determine how an individual compound could enhance the activity of a mixture of compounds in a plant extract. We first report that blends of *P. ribesioides* extracts and thymol do exhibit enhanced activity that is synergistic or additive against *S. exigua* larvae. However, the activity is an extract- and dose-specific. For instance, thymol generally enhanced the activity of hexane extract; however, the reaction was antagonistic if the hexane extract level was higher (LD_{30}) than the thymol level (LD_{10}). In contrast, although CH_2Cl_2 extract was moderately toxic, the combination of CH_2Cl_2 extract with thymol was more toxic than either component alone.

Similarly, ethyl EtOAc extract has been reported to be the most toxic fraction against *S. exigua* larvae [8], which was also found in the present study; synergistic activity was observed if the extract and thymol were combined at similar levels (1:1 thymol:EtOAc extract at the LD_{10} or LD_{30}). MeOH extract alone had moderate insecticidal activity, but all combinations with thymol were synergistic as binary mixtures. Moreover, this study found active ingredients of EtOAc extract as piperine, phenethylcinnamamide and cinnamic acid.

When combined with thymol, these compounds showed synergistic action at specific combination doses. It shows that the interactions between different compounds from a plant source can be synergistic or antagonistic, and the interaction could depend on the content, concentration, interaction between the compounds, and susceptibility of the target organism [33]. When a mixture of compounds is combined with a secondary metabolite, inactive compounds can influence the biological

Table 4 Impact on detoxification enzymes activity[1] in survived 2nd instar *S. exigua* after topical application of *P. ribesioides* extracts post-24 h treatment

Binary mixtures	CE activity	CF2	GST activity	CF2	AChE activity	CF2
Control	1.012^{d}	–	1.004^{de}	–	0.939^{de}	
Thymol (LD_{10}) + hexane extract (LD_{10}) 1.21 + 0.65	0.066^{a}	15.33	0.124^{b}	8.10	0.323^{c}	2.91
Thymol (LD_{30}) + hexane extract (LD_{10}) 9.54 + 0.65	0.106^{a}	9.55	0.074^{a}	13.57	0.026^{a}	36.12
Thymol (LD_{30}) + hexane extract (LD_{30}) 9.54 + 3.21	0.451^{bc}	2.24	0.324^{c}	3.10	0.227^{b}	4.14
Thymol (LD_{10}) + CH_2Cl_2 extract (LD_{30}) 1.21 + 1.73	0.923^{d}	1.10	0.676^{cd}	1.49	1.036^{de}	0.91
Thymol (LD_{30}) + CH_2Cl_2 extract (LD_{10}) 9.54 + 0.32	1.114^{d}	0.91	1.513^{e}	0.66	0.768^{d}	1.22
Thymol (LD_{30}) + CH_2Cl_2 extract (LD_{30}) 9.54 + 1.73	0.625^{c}	1.62	0.651^{cd}	1.54	2.061^{g}	0.46
Thymol (LD_{10}) + EtOAc extract (LD_{10}) 1.21 + 0.57	1.044^{d}	0.97	2.096^{f}	0.48	1.580^{f}	0.59
Thymol (LD_{30}) + EtOAc extract (LD_{30}) 9.54 + 1.60	1.947^{e}	0.52	1.691^{ef}	0.59	2.140^{g}	0.44
Thymol (LD_{10}) + MeOH extract (LD_{10}) 1.21 + 2.52	0.439^{b}	2.31	0.697^{cd}	1.44	0.917^{de}	1.02
Thymol (LD_{10}) + MeOH extract (LD_{30}) 1.21 + 39.74	0.661^{c}	1.53	1.082^{de}	0.93	1.141^{e}	0.82
Thymol (LD_{30}) + MeOH extract (LD_{10}) 9.54 + 2.52	1.040^{d}	0.97	0.821^{d}	1.22	0.443^{c}	2.12
Thymol (LD_{30}) + MeOH extract (LD_{30}) 9.54 + 39.94	1.117^{d}	0.91	0.911^{d}	1.10	1.974^{fg}	0.48
F-value	140.863		27.678		33.076	
P-value	< 0.0001		< 0.0001		< 0.0001	

1 Mean values within the same column followed by the same letter of each treatment are not significantly different ($P > 0.05$) by Tukey's test. The reaction of CE were measured as nM p-nitrophenol/min/mg; GST as nM glutathione conjugated product/min/mg; AChE as μM/min/mg of protein

2 CF is correction factor (detoxification enzyme activity of control / detoxification enzyme activity of treatment)

Table 5 Impact on detoxification enzymes activity[1] in survived 2nd instar *S. exigua* after topical application of isolated compounds of *P. ribesioides* extracts post-24 h treatment

Binary mixtures	CE activity	CF2	GST activity	CF2	AChE activity	CF2
Control	2.019^{e}	–	2.425^{d}	–	2.279^{d}	–
Piperine (LD_{10}) + thymol (LD_{30})	2.047^{e}	0.99	2.543^{d}	0.95	3.074^{e}	0.41
Piperine (LD_{30}) + thymol (LD_{10})	1.018^{c}	1.98	0.761^{bc}	3.19	1.714^{c}	1.33
Phenethylcinnamamide (LD_{10}) + thymol (LD_{10})	0.434^{a}	4.65	0.565^{ab}	4.29	1.021^{b}	2.23
Phenethylcinnamamide (LD_{30}) + thymol (LD_{10})	1.338^{d}	1.51	0.300^{a}	8.08	0.317^{a}	7.19
Cinnamic acid (LD_{10}) + thymol (LD_{10})	0.350^{a}	5.77	1.353^{c}	1.79	0.671^{ab}	3.40
Cinnamic acid (LD_{30}) + thymol (LD_{10})	0.667^{b}	3.03	1.120^{c}	2.17	0.342^{a}	6.66
F-value	431.95	–	111.67	–	174.701	–
P-value	0.062	–	0.017	–	0.000	–

1Mean values within the same column followed by the same letter of each treatment are not significantly different ($P > 0.05$) by Tukey's test. The reaction of CE were measured as nM p-nitrophenol/min/mg; GST as nM glutathione conjugated product/min/mg; AChE as μM/min/mg of protein

2CF is correction factor (detoxification enzyme activity of control / detoxification enzyme activity of treatment)

activity of the active secondary compounds. Our results similar to Silva et al. [34] that showed the binary mixtures (deltamethrin and linalool) caused mortality higher than the products applied alone at 100% LD_{50}, except to 75% LD_{50} deltamethrin added to 25% LD_{50} linalool, whose mortality did not differ the products alone which mean

linalool is a potential insecticidal and can be associated with pyrethroids to control of *S. frugiperda.*

Thus, the combination of major and minor compounds in an extract with thymol in the present case may have modified the activity by exerting significant synergistic or antagonistic effects. In conclusion, the synergism of a plant extract and a secondary metabolite depends on the composition of the extract and the specific doses used. This results will be an useful data for develop the highest efficiency in commercial level which is the main goal for this research. Further studies are required evaluate the synergistic combinations against field populations of *S. exigua.*

Xenobiotics are recognized by a variety of membrane-bound or nuclear receptors [35]. Detoxification enzymes, Cytochrome P 450, CE or GST in insects is generally demonstrated as the enzymatic defense against xenobiotic compounds and play significant roles in maintaining their normal physiological function. For instance, CE detoxify xenobiotics and contribute to several metabolic reactions, such as neurogenesis and developmental regulation induced by organophosphates, carbamates, and pyrethroids, which in turn are associated with insecticide resistance [36]. However, it is well known that multicomponent allelochemical mixtures can negate such impacts in extracts or in binary mixtures with almost no chance of resistance development [5].

Our present study showed that the combinations of thymol + MeOH or thymol + hexane extract act synergistically compared to the compound alone can also inhibit CE and GST suggesting that resistance was significantly delayed. Especially, thymol + hexane extract mostly inhibited for almost combination rate.

Moreover, our study found that that when used isolated compounds at LD30 dose with thymol, the results showed significantly inhibited all CE and GST level after binary mixture treatment of *S. exigua* larvae.

This again suggests that the impacts of the various treatments were both extract- compound- and dose-dependent, which further support the idea that the interaction depends on the content, concentration, interaction between the compounds and susceptibility of the target organism. The variability could also have been due to the configurations of GST, which comprise six major subclasses: sigma, omega, zeta, theta, delta, and epsilon. These enzymes play a crucial role in detoxification in innate cells and artificial molecules in culture systems [37].

AChE is an enzyme that catalyzes the hydrolysis reaction of acetylcholine. Xenobiotics and chemical pesticides inhibit these enzymes and cause intolerable accumulation of acetylcholine, resulting in acute and chronic neurotoxic symptoms [38, 39]. However, the extracts alone did not significantly affect AChE activity in the present study, but combinations with thymol moderately inhibited AChE. However, the combination of isolated compounds showed a significant inhibited AChE level; exceptional piperine + thymol (LD_{30}) induced the enzymatic activity. This finding further supports the idea that the possibility of resistance is reduced to a large extent when plant products are used in binary mixtures. It is known that essential oils that are mixtures of terpenes show intense insecticidal activity [10]. Therefore, there must be synergistic effects among these terpenes. However, the possibility of an impact on detoxification enzymes is variable. In other words, an insect is expected to detoxify a fraction of the LD_{50} of the major terpene in a mixture; in contrast, a minor terpene should act unimpeded, showing a lower LD_{50} than when acting alone. This metabolic model [40] has been applied to *M. domestica,* in which the synergism of piperonyl butoxide (PBO) with lethal concentrations of terpenes as fumigants has been established. The model indicates that the insect detoxifies the major component in a mixture. At the same time, the compounds in lesser proportions act as toxicants with higher toxicity than when assayed alone. This hypothesis seems especially pertinent in the present case because thymol synergized differently with the different extracts, which could have depended on the metabolite in the extract and the application dose.

From previous research, CE are one of detoxification enzymes are involved in resistance to pyrethroids and organophosphate in many insects [41–43]. Delorme et al [41] described that the *S. exigua* resistant strain has an enhanced esterase activity toward chromogenic substrates, such as naphthyl acetate or methylumbelliferyl acetate, the level depending on the substrate used. It is likely that activity toward chromogenic substrates and the hydrolysis of deltamethrin are related. Thus, CE play an important role in insect defense through catabolism of the esters of higher fatty acids that influence flight as well in the degradation of inert metabolic esters and various xenobiotics, including insecticides [43]. Changes in the activities of these enzymes are reflected in insect resistance to insecticides as well as in degradation of toxic molecules produced. GST plays a pivotal role in detoxification and cellular antioxidant defenses against oxidative stress by conjugating reduced glutathione to the electrophilic centers of natural and synthetic exogenous xenobiotics, including insecticides, allelochemicals, and endogenously activated compounds [43].

However, this research did not analyze CYP450 which is one of other important detoxification enzymes. This need to be concerned for further analysis. Additional

similar studies will be necessary to design effective insecticides based on natural plant mixtures and a synergistic compound.

Conclusion

When used at sublethal doses, binary mixtures of *P. ribesioides* extracts and thymol showed enhanced activity against *S. exigua* larvae. A metabolic model is suggested in which an insect may detoxify the major component in a mixture. At the same time, minor compounds could act as potential toxicants with higher toxicity than when assayed alone. The basis for this hypothesis is that the synergistic effects of thymol with the components in the extracts were variable. They depended on the metabolite stereochemistry in the extract and the dose of application. Such studies will help design effective insecticides based on combinations of natural plant mixtures with a synergistic compound.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-022-00306-2.

Additional file 1: Data S1. Sublethal dosages (LD_{10} and LD_{30} values) of various extracts from *P. ribesioides* twig powder and thymol against 2nd instar *S. exigua* larvae via topical application after 24 h post-treatment. **Data S2.** Toxicity (LD_{50} and LD_{90} values, µg/larva) of various compounds isolated from *P. ribesioides* ethyl acetate extract against 2nd instar *S. exigua* larvae via topical application after 24 and 48 h post-treatment. **Data S3.** Sublethal dosages (LD_{10} and LD_{30} values) of isolated compounds from *P. ribesioides* extract and against 2nd instar *S. exigua* larvae via topical application after 24 h post-treatment.

Acknowledgements

N.K. thanks Zoology Department and Chemistry Department, Faculty of Science, Kasetsart University for support and use of analytical equipment for this research. Thanks are also due to Rajamangala University of Technology Thanyaburi for help during this investigation. VB and NK thanks to postdoctoral research fund from KURDI, Kasetsart University. This work was financially supported by the Office of the Ministry of Higher Education, Science, Research and Innovation; and the Thailand Science Research and Innovation through the Kasetsart University Reinventing University Program 2021 (Activity No.2.2 Collaborating Professor-Online Program).

Author contributions

NK: conceptualization, procedure, investigation, writing—original draft, project administration. VB: conceptualization, procedure, investigation and project administration. WM: visualization. SN: isolation of allelochemicals. WP: plant extraction TY: visualization. NP: statistical advice. BD: statistical analysis. OK: writing—review and editing. YK: editing and review. All authors read and approved the final manuscript.

Funding

This work was financially supported by the Office of the Ministry of Higher Education, Science, Research and Innovation; and the Thailand Science Research and Innovation through the Kasetsart University Reinventing University Program 2021 (Activity No.2.2 Collaborating Professor-Online Program). This work was financially supported by postdoctoral research fund from KURDI, Kasetsrat University and funding from Zoology Department, Faculty of Science, Kasetsart for supporting English edit service fee.

Availability of data and materials

All data are presented in this article.

Declarations

Ethics approval and consent to participate

All bioassays in this article were performed with the approval of an appropriate animal ethics committee of Kasetsart University, Bangkok, Thailand.

Consent for publication

This article has been confirmed for publication in the journal.

Competing interests

The authors have no conflicts of interest.

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Received: 13 February 2022 Accepted: 31 May 2022
Published online: 08 June 2022

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