

Acute Toxicity, Synergistic and Antagonistic Effects of 12 Monoterpenoids Against the *Poratrioza sinica* Yang et Li (Hemiptera: Psyllidae)

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ABSTRACT

Poratrioza sinica (Hemiptera: Psyllidae) is a highly destructive pest that infests of wolfberry. To screen biodegradable and safe insecticides, 12 representative monoterpenoids in essential oils were evaluated for their acute toxicity, synergistic or antagonistic effects on adult *P. sinica*. The modified residual film method was used to test the toxicity of individual and binary mixture of these monoterpenoids against adult *P. sinica*. The effects of these monoterpenoids on acetylcholinesterase (AChE) and glutathione S-transferase (GST) activities in *P. sinica* were assessed *in vitro*. Correlation between numbers of synergistic or antagonistic binary mixtures and AChE or GST activities was analyzed. The results showed that 2-ethylimidazole had the strongest acute toxicity against *P. sinica* adult, with a median lethal concentration (LC₅₀) value of 0.52 g/L. Among the 66 binary mixtures, 19 showed strong synergistic effects, while 21 showed antagonistic effects. The most profound synergistic effect was the mixture of l-carvone and dihydrocarvone, with an expected mortality of 35.2% and actual mortality of 98.4%. Estragole had the highest frequency of antagonistic effect (7 combinations), and the most significant antagonism was observed when combining β-pinene and estragole, with an expected mortality of 29.0% and actual mortality of 3.8%. Furthermore, AChE inhibition was observed with estragole, cuminaldehyde, and 1,8-cineole displayed high potency. L-carvone showed the highest GST inhibition activity, followed by cuminaldehyde. Pearson correlation analysis revealed a significant negative correlation between GST inhibition rate and number of antagonistic binary mixtures. In conclusion, our findings suggest that 2-ethylimidazole and cuminyl alcohol have high toxicity against *P. sinica*. L-carvone was the best synergist, and binary mixtures of l-carvone with dihydrocarvone, cuminaldehyde, cuminyl alcohol, d-carvone, and estragole showed potential as control agents against *P. sinica*. This study provides insights for identifying safe and biodegradable insecticides and potential solutions for controlling *P. sinica*.

Article Information

Received 22 October 2021

Revised 20 March 2023

Accepted 17 May 2023

Available online 20 June 2023

(early access)

Published 16 December 2023

Authors' Contribution

Conceptualization, methodology: WW and L-QD. Investigation: S-JW, QS. Data curation, formal analysis, writing original draft preparation: WW. Supervision, software, validation: X-LW. Validation, writing reviewing and editing: L-QD. All authors have read and agreed to the published version of the manuscript.

Key words

Monoterpenoids, *Poratrioza sinica*, Binary mixtures, Acute toxicity, Synergistic effect, AChE inhibition, GST inhibition

INTRODUCTION

Poratrioza sinica Yang et Li (Hemiptera: Psyllidae) is a destructive pest that infests Chinese wolfberry (*Lycium barbarum*) in the northwest regions of China. This insect species feeds on the sap of young leaves, shoots, buds and fruits, causing premature leaf drop, diminish plant growth, and reduce fruit quality and yield when the population

density is too high. In addition, the honeydew secreted by *P. sinica* promotes the growth of sooty blotch on leaves and fruits. Currently, controlling *P. sinica* mainly relies on extensive use of synthetic insecticides. However, the use of synthetic insecticides has brought several serious problems, including negative impacts on environment and non-target organisms, such as humans (Hodgson and Levi, 1996; Singh *et al.*, 2012), as well as development of resistant *P. sinica* populations. These issues have driven the search for environmentally safe alternative control measures. Amongst alternative strategies aimed at reducing insect populations, the use of essential oils is a promising strategy.

Essential oils are secondary metabolites of plants that possess significant biological activity and various pesticidal effects (Abdelfattah *et al.*, 2018; Hennia *et al.*, 2019), including insecticidal activity (Ebadollahi *et al.*, 2021; Said-Al Ahl *et al.* 2017). Despite numerous

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0030-9923/2024/0001-0225 \$ 9.00/0



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studies demonstrating their insecticidal effects, only a few essential oil insecticides are commercially produced (Isman and Grieneisen, 2014) due to limited production, quality and quantity issues, and high prices of some essential oils. These factors have hindered the production and wider expansion of essential oil insecticides.

The insecticidal properties of essential oils are primarily attributed to their main active constituents. Thymol is the main insecticidal active ingredient in essential oils extracted from *Trachyspermum ammi*, and has been found to be effective against *Aethina tumida* (Bisrat and Jung, 2020). Moreover, dihydrocarvone, carvone and cuminaldehyde are active constituents of essential oils extracted from *Anethum graveolens*, *Cuminum cyminum* and *Carum carvi* that have shown efficacy against *Sitophilus oryzae* adults and *Aedes albopictus* larvae (Kim *et al.*, 2013; Seo *et al.*, 2015). Essential oils extracted from *Erechtites hieraciifolius* and *E. valerianifolius* have been shown to possess good mosquito larvicidal properties, attributed to the presence of limonene and α -pinene in the essential oil of *E. hieraciifolius*, as well as α -pinene, β -caryophyllene, and myrcene in essential oil of *E. valerianifolius* (Hung *et al.*, 2019). Recent research suggests that many of the active substances contained in essential oils can be produced synthetically with high quality and at a lower cost. However, the use of a single active substance with a single mechanism of action could contribute to the development of resistant insect populations, as observed with other synthetically produced insecticides (Ranson *et al.*, 2009). In contrast, essential oils contain complex mixtures of active substances with different mechanisms of action, which may prevent the development of resistance in insects, thus providing a major advantage of essential oils as insecticides (Regnault-Roger *et al.*, 2012; Sutthanont *et al.*, 2010). Individual components contained in essential oils can exhibit diverse synergistic or antagonistic effects, which significantly influencing their biological efficacy (Hummelbrunner and Isman, 2001; Pavela, 2008, 2014). Therefore, a thorough understanding of this phenomenon is essential for developing essential oils insecticides with standardized mixtures and declared activity, while maintaining relatively lower costs.

Acetylcholinesterase (AChE) is a crucial enzyme that helps break down acetylcholine into choline and acetate at the neuromuscular junction. The choline produced by AChE activity is recycled by being transported back to the presynaptic neuron for the synthesis of new acetylcholine. Inhibition of AChE activity may hinder neurotransmission, ultimately leading to insect death (López and Pascual-Villalobos, 2010). Several essential oils from aromatic plants and monoterpenes have been identified as inhibitors

of AChE isolated from different insect species (Abdelgaleil *et al.*, 2009; Kim *et al.*, 2013). Glutathione-S-transferase (GST) functions in the detoxification of foreign substances by conjugating glutathione (GSH) with electrophilic molecules. It plays a crucial role in detoxifying harmful compounds and developing insecticide resistance (Cisse *et al.*, 2017; Li *et al.*, 2019; Piccoli *et al.*, 2019).

In this study, to gain a better understanding of the mutual relationships between essential oil compounds, we selected 12 representative monoterpenoids found in essential oils. These compounds were tested individually and as binary mixtures for their acute toxicity against *P. sinica*. This will facilitate an improved understanding of the general principles of the mutual relationships of essential oil compounds and determine a suitable mixture of active substances for developing new essential oil insecticides against *P. sinica*. Furthermore, the effects of these 12 monoterpenoids on the activity of AChE and GST of *P. sinica* were assessed *in vitro* to explore the action mechanism of the monoterpenoids.

MATERIALS AND METHODS

Chemicals

Dihydrocarvone (98%) was obtained from Sigma-Aldrich (Saint Louis, MO, USA); L-carvone (98%) and estragole (98%) was obtained from Alfa Aesar (Beijing, China); 2-ethylimidazole (2-MIM, 99%), β -caryophyllene (BCP, 80%), cuminaldehyde (97%), acetylthiocholine iodide (ATCI), and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from RHAWN (Shanghai, China); cuminyl alcohol (97%) was procured from Xiya Reagent (Shandong) Co. Ltd. (Linyi, China); 1,8-cineole (99%) and d-carvone (98%) were obtained from Energy Chemical (Shanghai, China); limonene (97%) were obtained from Macklin Biochemical Co., Ltd. (Shanghai, China); α -pinene (95%) and β -pinene (95%) were procured from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China); acetone, alcohol, NaH_2PO_4 , and Na_2HPO_4 were purchased from the Tianjin Chemical Reagent Factory (Tianjin, China); Coomassie brilliant blue G-250 and bovine serum albumin were procured from Amresco (Solon, OH, USA); the glutathione S-transferase (GST) assay kits were purchased from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China).

Plants and insects

The first generation of *Poratrioza sinica* adult was initially sourced from the Science and Technology Garden of Inner Mongolia Agricultural University (Hohhot, China), and subsequently raised in a laboratory environment without exposure to any insecticides. Chinese

wolfberry (*Lycium barbarum*) seedlings were used as their food source during this process.

Chinese wolfberry seedlings were cultivated in plastic pots with dimensions of 15 cm height and 10 cm diameter. The pots were filled with a mixture of peat soil, perlite, and vermiculite in a 60:20:20 ratio, with a pH range of 6–7. The pots were then placed inside cages covered with an insect-proof netting of 80 mesh size (270 × 170 × 240 cm). The growth conditions were maintained at room temperature (21–26°C) under a photoperiod of 16:8 h (L:D), regulated using a timing socket. Seedlings that grew to be 25–30 cm tall were utilized for subsequent experiments.

To breed *P. sinica*, two pots (containing one seedling for each pot) were transferred into a small insect-proof and net-covered cage measuring 35 cm × 35 cm × 45 cm with a 120 mesh size. Subsequently, 20 pairs of three-day-old *P. sinica* adults were introduced into this cage and removed two days later to ensure hatching of eggs at approximately the same time. The cage was incubated under the same laboratory temperature and photoperiod conditions as described above.

Acute toxicity assessment

To evaluate the acute toxicity of 12 monoterpenoids to *P. sinica*, the modified residual film method was employed as previously described (Shotkoski *et al.*, 1990; Shufran *et al.*, 1997). The 12 monoterpenoids examined were 2-ethylimidazole, estragole, cuminyl alcohol, d-carvone, l-carvone, cuminaldehyde, dihydrocarvone, β-caryophyllene, β-pinene, 1,8-cineole, α-pinene, and limonene. Serial dilutions of each monoterpenoid were prepared in acetone to generate at least ten concentrations (0.06, 0.12, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 mL/L), except for 2-ethylimidazole (solid, with the unit of measurement being g/L). Then, 500 μL of each dilution was added into a glass tube (10 cm length, 1.5 cm diameter), and slowly rolled on a table to ensure the formation of a residual film on the inner surface of the tube until the acetone had completely evaporated. Simultaneously, a piece of wolfberry leaf was immersed in the same solution for 5 s, and then dried on filter paper. The leaf was subsequently transferred into the tube containing the residual film formed by the same tested solution.

Then, 20 *P. sinica* adults (newly emerged about 3 days) were released into each prepared test tube, and the mortality was recorded after 24 h of exposure. The tube was sealed with Parafilm (PM-996, Bemis, Neenah, WI, USA) to prevent their escape, three replicates were conducted, with n=20 *P. sinica* adults for each concentration, a total n= 60. The assays were performed in a growth chamber with a photoperiod of 16:8 h (L:D) at 26°C.

The values of median lethal concentration (LC₂₅,

LC₅₀, LC₉₀), confidence interval of 95% (CI₉₅), slope, and χ^2/df were estimated using probit analysis (SPSS Statistics 22, IBM, New York, NY, USA). Differences among LC₂₅, LC₅₀ or LC₉₀ values were considered significant when their 95% CI did not overlap (Ebling *et al.*, 2004).

Assessment of AChE activity

The inhibitory effect of 12 monoterpenoids on AChE activity in *P. sinica* was evaluated *in vitro* using the modified Ellman's method (Ellman *et al.*, 1961). Another 30 healthy *P. sinica* adults were homogenized in an ice bath using a glass tissue grinder with pre-cooled 0.2 M phosphate buffer (PB, pH 7.0). The homogenate was then centrifuged at 10,000 g for 20 min at 4°C, and the collected supernatant was used as the enzyme solution for assessing AChE activity.

To determine the AChE activity, the tested monoterpenoids were diluted in acetone to a concentration of 20 mL/L. Next, 0.02 mL of the diluted monoterpenoid solution, 0.15 mL of the enzyme supernatant, and 0.53 mL of PB (pH 7.0, 0.2 mol/L) were mixed in a tube. After 5 min, 0.2 mL of 0.03 mol/L ATCI was added, and the mixture was incubated at 30°C for 15 min. Then 2.1 mL of 0.125 mmol/L DTNB was added, and after 2 min, the absorbance (OD at 412 nm) was measured using a TU-1810 UV-visible spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., Beijing, China). Three biological replicates were performed for each monoterpenoid, with acetone being used as the control and the dead enzyme (inactivated in boiling water) as a blank. The inhibition rate was calculated as follows:

$$\text{Inhibition rate (\%)} = 100 - (\text{Treatment OD} - \text{Blank OD}) / (\text{Control OD} - \text{Blank OD}) \times 100$$

Assessment of GST activity

To assess the inhibitory effect of 12 monoterpenoids on the GST activity of *P. sinica*, another 30 healthy adults of *P. sinica* were homogenized using a glass tissue grinder in an ice bath with pre-cooled PB (pH 7.0, 0.2 mol/L). The resulting homogenate was centrifuged at 8000 g for 10 min at 4°C, and the supernatant was collected as the crude enzyme solution for analysis of GST activity. The GST activity was calculated following the instruction of the GST assay kits.

Acute toxicity of binary mixtures

To ascertain the antagonistic or synergistic effects of the 12 monoterpenoids, the mortalities of *P. sinica* caused by these monoterpenoids, both individually and in binary mixtures, using the modified residual film method illustrated above. The two monoterpenoids were combined in a 1:1 ratio volume (concentration in LC₂₅, values listed

in Table I). Each binary mixture was replicated 4 times and 20 adults of *P. sinica* for each replication. Expected mortalities of binary mixtures were calculated using the following equation (Hummelbrunner and Isman, 2001; Pavela, 2014, 2015):

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

where, E represents the expected mortality of binary mixtures; O_a and O_b are the observed mortalities of the pure monoterpenoid A or B in the binary mixtures of A and B at the given concentration.

The χ^2 comparisons analysis was utilized to designate the effects of binary mixtures as either antagonistic, additive, or synergistic, using the equation described below:

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

where, O_m is the observed mortality of binary mixtures; E is the expected mortality; $\chi^2 = 3.84$ with $df = 1$ at $p = 0.05$. If $\chi^2 > 3.84$ and $O_m > E$, it was perceived as synergistic; if $\chi^2 > 3.84$ and $O_m < E$, it was perceived as antagonistic; if $\chi^2 \leq 3.84$, it represented additive effects.

Statistical analysis

Data are presented as mean \pm standard deviation (SD). To compare the inhibitory rates of different monoterpenoids on AChE and GST activity, we used one-way analysis of variance (ANOVA) (Duncan's test) at $p < 0.05$. Pearson's correlation coefficient was used to analyze

the relationship between the synergistic or antagonistic effects of monoterpenoids binary mixtures and AChE or GST inhibition rates using SPSS software.

RESULTS

Acute toxicity of 12 monoterpenoids

The variability in the toxicities of 12 monoterpenoids against *P. sinica* adults were shown in Table I. At the highest concentration tested (10 mL/L), 2-ethylimidazole, estragole, cuminyl alcohol, d-carvone, l-carvone, cuminaldehyde and dihydrocarvone caused 100% mortality, whereas β -caryophyllene resulted in 88.3% mortality. In contrast, 1,8-cineole, β -pinene, and α -pinene led to less than 50% mortality, with limonene resulting in only 5% mortality. Among these monoterpenoids, 2-ethylimidazole demonstrated the highest toxicity to *P. sinica* adults, with the lowest LC_{25} , LC_{50} and LC_{90} values. The LC_{50} values of estragole, cuminyl alcohol and d-carvone were 2.11, 2.17, and 2.28 mL/L, respectively, with no significant differences in their lethal activities. Similarly, l-carvone, cuminaldehyde, dihydrocarvone and β -caryophyllene exhibited LC_{50} values of 3.06, 3.17, 3.19, and 3.31 mL/L, respectively, with no significant variations in their lethal activities. As for β -pinene, 1,8-cineole, α -pinene and limonene, their LC_{50} values were estimated to be higher than 10 mL/L, as their mortality was less than 50% at the highest concentration tested (10 mL/L).

Table I. Toxicities of 12 monoterpenoids against *P. sinica* adults.

Monoterpenoid	Mortalities (%) at 10 mL/L	LC_{25} (CI_{95}) (mL/L)	LC_{50} (CI_{95}) (mL/L)	LC_{90} (CI_{95}) (mL/L)	Slope	χ^2/df
2-ethylimidazole	100.0 \pm 0.0	0.24 (0.17–0.31)a	0.52 (0.41–0.66)a	2.31 (1.63–3.84)a	3.57	34.4/20
Estragole	100.0 \pm 0.0	1.36 (1.16–1.55)b	2.11 (1.88–2.36)b	4.82 (4.05–6.15)bc	5.03	12.9/13
Cuminyl alcohol	100.0 \pm 0.0	1.74 (1.56–1.89)c	2.17 (2.01–2.35)b	3.29 (2.94–3.95)a	7.08	18.3/13
D-carvone	100.0 \pm 0.0	1.62 (1.39–1.82)c	2.28 (2.04–2.54)b	4.34 (3.73–5.40)b	3.66	43.3/22
L-carvone	100.0 \pm 0.0	2.25 (2.01–2.46)d	3.06 (2.83–3.28)c	5.47 (4.97–6.21)c	5.07	19.6/18
Cuminaldehyde	100.0 \pm 0.0	2.08 (1.63–2.45)d	3.17 (2.71–3.67)c	7.10 (5.79–9.73)e	4.57	19.9/13
Dihydrocarvone	100.0 \pm 0.0	2.34 (2.11–2.55)d	3.19 (2.95–3.44)c	5.77 (5.15–6.61)c	2.46	9.6/16
β -caryophyllene	88.3 \pm 2.9	1.76 (1.45–2.06)c	3.31 (2.89–3.79)c	11.00 (9.00–14.28)f	1.98	7.9/19
β -pinene	36.7 \pm 5.8	8	>10	>10	-	-
1,8-cineole	16.7 \pm 2.9	8	>10	>10	-	-
α -pinene	16.7 \pm 7.6	8	>10	>10	-	-
Limonene	5.0 \pm 5.0	8	>10	>10	-	-

Notes: Serial dilutions of each monoterpenoid were prepared in acetone to generate at least six concentrations (0.06, 0.12, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 mL/L), except for 2-ethylimidazole (solid, with the unit of measurement being g/L). LC_{25} , LC_{50} , and LC_{90} represent the dose necessary to kill 25, 50 and 90% of *P. sinica* adults, respectively; CI_{95} : 95% confidence interval. Differences among LC values were considered significant when their 95% CI did not overlap by 50%. The lowercase letters in the same column represent significant differences.

The slope value indicated the difference of individual sensitivities of *P. sinica* population to the tested solutions. The larger the slope value, the lesser difference in individual sensitivities of the population (Hughes *et al.*, 1984). The difference of individual sensitivities of *P. sinica* population to cuminyl alcohol were lesser than that to other monoterpenoids. The results indicated that 2-ethylimidazole was the most acutely toxic monoterpene to *P. sinica*.

In vitro inhibitory effects of 12 monoterpenoids on the activities of AChE and GST in *P. sinica*

In vitro experiments were conducted to examine the effects of 12 monoterpenoids on the activities of AChE and GST of *P. sinica*. The inhibition rates for AChE varied among the tested monoterpenoids, with estragole displaying the highest inhibition rate of 95.0%, followed by cuminaldehyde (91.4%) and 1,8-cineole (88.0%). The remaining monoterpenoids such as α -pinene (74.2%), d-carvone (66.9%), l-carvone (42.0%), and dihydrocarvone (35.2%) showed lower inhibition rates. Conversely, β -caryophyllene, cuminyl alcohol, 2-ethylimidazole, limonene and β -pinene demonstrated rates under 15% (Fig. 1). It can be inferred that the activity of AChE was significantly inhibited by estragole, cuminaldehyde, and 1,8-cineole.

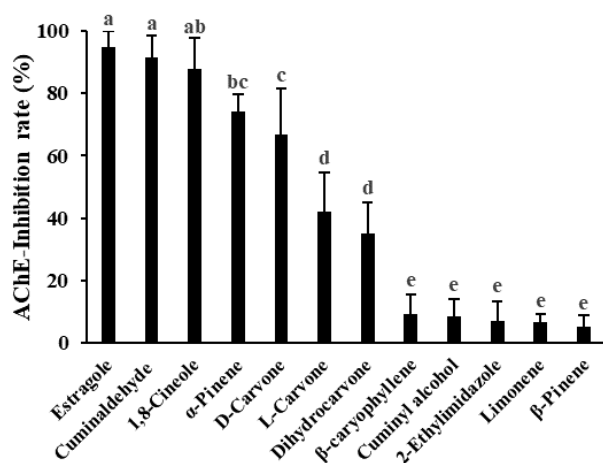


Fig. 1. Inhibition rates of 12 monoterpenoids to AChE activity of *P. sinica* *in vitro*.

Notes: The concentration of all 12 monoterpenoids tested was 20 mL/L. Data are presented as number or mean \pm SD. Columns with the same letters on top are not significantly different based on One-way ANOVA at $p < 0.05$, Duncan's test.

Regarding the GST activities of *P. sinica*, 12 monoterpenoids also showed varied inhibition rates (Fig.

2). L-carvone exhibited the highest inhibition rate of 65.4%, followed by cuminaldehyde (58.8%), β -pinene (51.0%), dihydrocarvone (47.2%), 1,8-cineole (46.7%), cuminyl alcohol (41.7%), β -caryophyllene (41.6%), d-carvone (39.0%), α -pinene (35.9%), estragole (28.5%), 2-ethylimidazole (27.6%) and Limonene (22.6%). L-carvone and cuminaldehyde showed the highest GST inhibition activity.

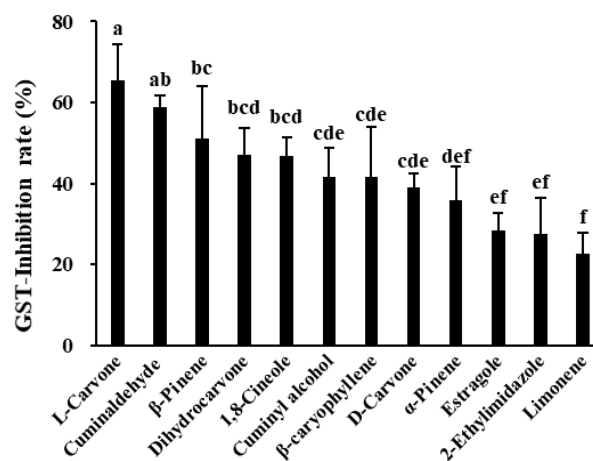


Fig. 2. Inhibition rates of 12 monoterpenoids to GST activity of *P. sinica* *in vitro* at the concentration of 20 mL/L.

Notes: Data are presented as number or mean \pm SD. Columns with the same letters on top are not significantly different based on One-way ANOVA at $p < 0.05$, Duncan's test.

Toxicity of binary mixtures against *P. sinica* adults

A total of 66 binary mixtures were tested for their acute toxicity against *P. sinica* adults (Table II), of which 19 combinations exhibited strong synergistic effects, 21 showed significant antagonistic effects, and 26 displayed additive effects. A higher χ^2 value indicated a stronger synergistic or antagonistic effects. The most profound synergistic effects were observed with the following binary mixtures: dihydrocarvone and l-carvone ($\chi^2 = 113.6$), l-carvone and cuminaldehyde ($\chi^2 = 108.3$), l-carvone and cuminyl alcohol ($\chi^2 = 109.4$), estragole and l-carvone ($\chi^2 = 112.3$). While, limonene and dihydrocarvone ($\chi^2 = 16.8$), α -pinene and dihydrocarvone ($\chi^2 = 15.4$), α -pinene and estragole ($\chi^2 = 19.5$), β -pinene and cuminaldehyde ($\chi^2 = 18.1$) exhibited the most profound antagonistic effects. The highest mortalities were achieved for the combinations: dihydrocarvone and l-carvone (98.4%), l-carvone and cuminaldehyde (96.5%), l-carvone and cuminyl alcohol (96.4%), estragole and l-carvone (93.3%), d-carvone and l-carvone (98.5%).

Table II. Effect of binary mixtures of 12 monoterpenoids prepared as LC₂₅ combinations on mortality against *P. sinica*.

Monoterpenoid A	Monoterpenoid B	Mortality (%)				χ^2	Effects
		Pure monoterpenoids		Binary mixtures			
		Observed A	Observed B	Expected	Observed		
1,8-cineole	Limonene	3.6	1.8	5.4	9.2	2.7	Additive
1,8-cineole	α -pinene	3.6	5.5	9.0	26.3	33.6	Synergistic
1,8-cineole	β -pinene	3.6	13.7	16.9	9.2	3.5	Additive
1,8-cineole	Dihydrocarvone	3.6	20.8	23.7	17.7	1.5	Additive
1,8-cineole	Estragole	3.6	17.8	20.8	6.1	10.4	Antagonistic
1,8-cineole	D-carvone	3.6	33.6	36.0	28.2	1.7	Additive
1,8-cineole	L-carvone	3.6	18.2	21.1	23.3	0.2	Additive
1,8-cineole	Cuminaldehyde	3.6	20.5	23.4	15.5	2.7	Additive
1,8-cineole	Cuminy alcohol	3.6	21.1	24.0	5.7	13.9	Antagonistic
1,8-cineole	2-ethylimidazole	3.6	18.4	21.4	5.7	11.6	Antagonistic
1,8-cineole	β -caryophyllene	3.6	17.2	20.2	12.5	3	Additive
Limonene	α -pinene	1.8	5.5	7.2	13.3	5.2	Synergistic
Limonene	β -pinene	1.8	13.7	15.2	17.9	0.5	Additive
Limonene	Dihydrocarvone	1.8	20.8	22.2	2.9	16.8	Antagonistic
Limonene	Estragole	1.8	17.8	19.2	4.2	11.8	Antagonistic
Limonene	D-carvone	1.8	33.6	34.8	25	2.7	Additive
Limonene	L-carvone	1.8	18.2	19.6	23.3	0.7	Additive
Limonene	Cuminaldehyde	1.8	20.5	22.0	11.8	4.7	Antagonistic
Limonene	Cuminy alcohol	1.8	21.1	22.5	6.5	11.5	Antagonistic
Limonene	2-ethylimidazole	1.8	18.4	19.9	13.3	2.2	Additive
Limonene	β -Caryophyllene	1.8	17.2	18.7	15.8	0.5	Additive
α -pinene	β -pinene	5.5	13.7	18.5	13.2	1.5	Additive
α -pinene	Dihydrocarvone	5.5	20.8	25.2	5.5	15.4	Antagonistic
α -pinene	Estragole	5.5	17.8	22.3	1.5	19.5	Antagonistic
α -pinene	D-carvone	5.5	33.6	37.3	27.9	2.3	Additive
α -pinene	L-carvone	5.5	18.2	22.7	23.1	0	Additive
α -pinene	Cuminaldehyde	5.5	20.5	24.9	15.4	3.6	Additive
α -pinene	Cuminy alcohol	5.5	21.1	25.5	15	4.3	Antagonistic
α -pinene	2-ethylimidazole	5.5	18.4	22.9	30.3	2.4	Additive
α -pinene	β -caryophyllene	5.5	17.2	21.8	5.2	12.6	Antagonistic
β -pinene	Dihydrocarvone	13.7	20.8	31.7	25.1	1.4	Additive
β -pinene	Estragole	13.7	17.8	29	3.8	22	Antagonistic
β -pinene	D-carvone	13.7	33.6	42.7	45.1	0.1	Additive
β -pinene	L-carvone	13.7	18.2	29.4	47.5	11.2	Synergistic
β -pinene	Cuminaldehyde	13.7	20.5	31.4	7.6	18.1	Antagonistic
β -pinene	Cuminy alcohol	13.7	21.1	31.9	40.1	2.1	Additive
β -pinene	2-ethylimidazole	13.7	18.4	29.6	11.9	10.6	Antagonistic

Table continued on next page.....

Monoterpenoid A	Monoterpenoid B	Mortality (%)				χ^2	Effects
		Pure monoterpenoids		Binary mixtures			
		Observed A	Observed B	Expected	Observed		
β -pinene	β -caryophyllene	13.7	17.2	28.6	30	0.1	Additive
Dihydrocarvone	Estragole	20.8	17.8	34.9	12.6	14.3	Antagonistic
Dihydrocarvone	D-carvone	20.8	33.6	47.4	84.6	29.1	Synergistic
Dihydrocarvone	L-carvone	20.8	18.2	35.2	98.4	113.6	Synergistic
Dihydrocarvone	Cuminaldehyde	20.8	20.5	37.1	80.4	50.5	Synergistic
Dihydrocarvone	Cuminyl alcohol	20.8	21.1	37.5	62.8	17	Synergistic
Dihydrocarvone	2-ethylimidazole	20.8	18.4	35.4	22.3	4.8	Antagonistic
Dihydrocarvone	β -caryophyllene	20.8	17.2	34.5	32.3	0.1	Additive
Estragole	D-carvone	17.8	33.6	45.4	71.8	15.4	Synergistic
Estragole	L-carvone	17.8	18.2	32.7	93.3	112.3	Synergistic
Estragole	Cuminaldehyde	17.8	20.5	34.7	79.0	56.6	Synergistic
Estragole	Cuminyl alcohol	17.8	21.1	35.1	52.9	9.0	Synergistic
Estragole	2-ethylimidazole	17.8	18.4	32.9	18.8	6.1	Antagonistic
Estragole	β -caryophyllene	17.8	17.2	31.9	12.0	12.4	Antagonistic
D-carvone	L-carvone	33.6	18.2	45.7	98.6	61.4	Synergistic
D-carvone	Cuminaldehyde	33.6	20.5	47.2	87.8	34.8	Synergistic
D-carvone	Cuminyl alcohol	33.6	21.1	47.6	70.0	10.5	Synergistic
D-carvone	2-ethylimidazole	33.6	18.4	45.8	33.2	3.5	Additive
D-carvone	β -caryophyllene	33.6	17.2	45.0	42.3	0.2	Additive
L-carvone	Cuminaldehyde	18.2	20.5	35.0	96.5	108.3	Synergistic
L-carvone	Cuminyl alcohol	18.2	21.1	35.4	96.4	104.9	Synergistic
L-carvone	2-ethylimidazole	18.2	18.4	33.2	55.7	15.1	Synergistic
L-carvone	β -caryophyllene	18.2	17.2	32.2	18	6.3	Antagonistic
Cuminaldehyde	Cuminyl alcohol	20.5	21.1	37.3	65.8	21.8	Synergistic
Cuminaldehyde	2-ethylimidazole	20.5	18.4	35.2	28.5	1.3	Additive
Cuminaldehyde	β -caryophyllene	20.5	17.2	34.2	31.9	0.2	Additive
Cuminyl alcohol	2-ethylimidazole	21.1	18.4	35.7	75.2	43.9	Synergistic
Cuminyl alcohol	β -caryophyllene	21.1	17.2	34.7	15.1	11.1	Antagonistic
2-ethylimidazole	β -caryophyllene	18.4	17.2	32.5	20.1	4.7	Antagonistic

L-carvone displayed a synergistic effect with 7 monoterpenoids, while cuminyl alcohol exhibited this effect with 6 monoterpenoids. Cuminaldehyde and d-carvone displayed synergistic effects with 5 monoterpenoids each, and dihydrocarvone and estragole with 4 monoterpenoids each. In contrast, α -pinene, 2-ethylimidazole, limonene, 1,8-cineole, and β -pinene exhibited limited synergistic effects. Estragole, on the other hand, demonstrated antagonistic effects in 7 combinations. The maximal synergic binary mixtures were dihydrocarvone and l-carvone, l-carvone and cuminaldehyde, l-carvone and cuminyl alcohol, d-carvone and l-carvone, and estragole and l-carvone, suggesting that L-carvone could be

considered as the most effective synergist (Table III).

Correlation between the number of synergistic or antagonistic binary mixtures and AChE or GST activities

The five compounds, namely l-carvone, cuminaldehyde, β -pinene, 1,8-cineole, and cuminaldehyde, demonstrated a relatively high degree of inhibition against GST activity of *P. sinica in vitro*, and resulted in fewer antagonistic binary mixtures against *P. sinica* mortality. This indicates that there may be a correlation between the number of antagonistic binary mixtures and the GST inhibition ability. Pearson correlation analysis revealed a negative correlation trend between the GST inhibition rate

Table III. The number of monoterpenoids reaching the level of being a synergist or antagonist.

Monoterpenoid	Synergist ^a	Antagonist ^a	Additive ^a	Maximal synergistic effect ^b
L-carvone	7	1	3	Dihydrocarvone
Cumyl alcohol	6	4	1	L-carvone
D-carvone	5	0	6	L-carvone
Cuminaldehyde	5	2	4	L-carvone
Dihydrocarvone	4	4	3	L-carvone
Estragole	4	7	0	L-carvone
α -pinene	2	4	5	1,8-cineole
2-ethylimidazole	2	5	4	Cumyl alcohol
1,8-cineole	1	3	7	α -pinene
β -pinene	1	3	7	L-carvone
Limonene	1	4	6	α -pinene
β -caryophyllene	0	5	6	-

^a The number of the monoterpenoid creating the synergistic, antagonistic, or additive effect on mortality of *P. sinica*; ^b Monoterpenoid with which was achieved most significant synergism.

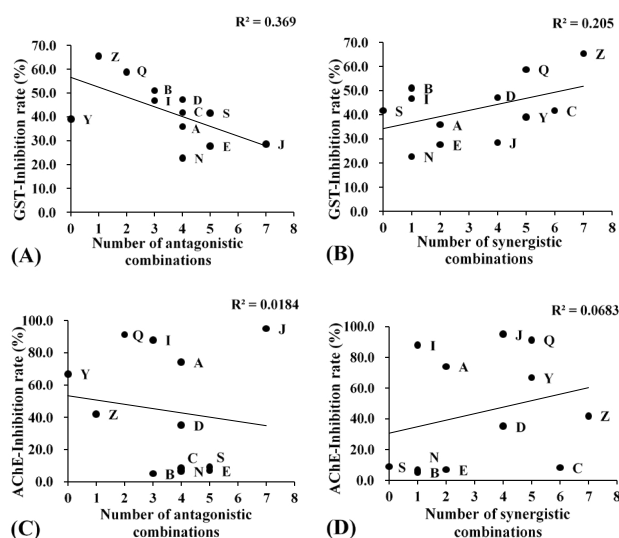


Fig. 3. Correlation analysis between AChE or GST inhibition rates and the number of synergistic or antagonistic combinations

Data analyzed using Pearson correlation coefficient at a significance level of $p < 0.05$. Z, L-carvone; C, Cumyl alcohol; Y, D-carvone; Q, Cuminaldehyde; D, Dihydrocarvone; J, Estragole; A, α -pinene; E, 2-ethylimidazole; I, 1,8-cineole; B, β -pinene; N, Limonene; S, β -caryophyllene.

and the number of antagonistic binary mixtures (Pearson coefficient = -0.608 , $R^2 = 0.369$, $p = 0.036$, Fig. 3A).

However, no significant relationship was found between the GST inhibition rates and the number of synergistic binary mixtures (Pearson coefficient = 0.453 , $R^2 = 0.205$, $p = 0.140$, Fig. 3B). Similarly, no significant correlation was observed between the AChE inhibition rates and the number of synergistic or antagonistic binary mixtures (Pearson coefficient = -0.136 , $R^2 = 0.018$, $p = 0.412$, Fig. 3C; Pearson coefficient = 0.261 , $R^2 = 0.068$, $p = 0.674$, Fig. 3D).

In brief, the inhibition of GST activity in *P. sinica* by certain compounds was found to be negatively correlated with the number of antagonistic binary mixtures, while no significant correlation was observed between AChE inhibition and the number of synergistic or antagonistic binary mixtures.

DISCUSSION

In this study, the toxicity of 12 monoterpenoids was tested against *P. sinica* adults, and the results showed that 2-ethylimidazole had the highest toxicity with the lowest LC_{25} , LC_{50} and LC_{90} values. However, there was limited reports on its insecticidal activity, and further research is needed to determine its pesticide effects and mechanisms. Cumyl alcohol, estragole, and d-carvone also demonstrated better toxicity against *P. sinica* with low LC_{50} and LC_{90} values. Other monoterpenoids, such l-carvone, cuminaldehyde, dihydrocarvone and β -caryophyllene were also efficient at eliminating *P. sinica*. In contrast, Limonene, α -pinene, β -pinene, and 1,8-cineole showed low mortality, even at the highest tested concentration of 10 mL/L.

It was noted that d-carvone and cumyl alcohol were more virulent than l-carvone and cuminaldehyde, respectively. This suggests that the molecular structure of these substances may influence their toxicity. However, the relationship between the efficacy of individual substances and their molecular structures was difficult to define (Pavela, 2015). Previous studies have shown that lipophilicity influenced the insecticidal activity of lipophilic compounds through enzyme inhibition (Ryan and Byrne, 1988; Santos *et al.*, 2010). For example, thymol and carvacrol, which have lipophilic CH chains outside a phenyl ring, displayed higher larvicidal activity against *Aedes aegypti* larvae than phenol alone (Santos *et al.*, 2010). Exocyclic double bonds have also been found to influence the toxicity of α -pinene and β -pinene to larvae of *A. aegypti* (Lucia *et al.*, 2007; Perumalsamy *et al.*, 2010; Simas *et al.*, 2007).

The insecticidal activities of essential oils and their constituents also depends on the type of insects. Previous study has shown that cuminaldehyde, cumyl alcohol,

1,8-cineole, limonene, and β -caryophyllene were toxic to *Stomoxys calcitrans*, with cuminaldehyde being the most effective (Hieu *et al.*, 2012). However, neither 1,8-cineole nor β -caryophyllene showed insecticidal activity against *P. sinica* in our study. Similarly, the toxicities of essential oils and their constituents against different insects varied. For instance, Yeom *et al.* (2015) found that estragole was more effective than β -caryophyllene against German cockroach (*Blattella germanica*), and our work also revealed that estragole outperformed β -caryophyllene in controlling *P. sinica*. In contrast, l-carvone demonstrated significant toxicity against *S. oryzae*, *Rhyzopertha dominica* adults and *Tribolium castaneum* adults in a previous study (Tripathi *et al.*, 2003), but it was not effective against *P. sinica* in our work.

Previous research has shown that mixing essential oil components or chemical pesticides may result in synergistic, antagonistic, or additive effects (Wu *et al.*, 2017). Synergistic effects of complex mixtures was important to standardize the formulations of insecticides (Akram *et al.*, 2023; Jabbar *et al.*, 2022), especially to essential oil insecticides. In our study, 66 binary mixtures were tested, of which 19 resulted in a synergistic effect, and 21 had an antagonistic effect on *P. sinica* mortality. Notably, despite using concentrations that matched the estimated LC_{25} in the tests, pure monoterpenoids often caused significantly lower mortality than expected (25%), but when combined, some mixtures created up to 95% mortality. This phenomenon was observed in 5 binary mixtures: l-carvone and cuminaldehyde, l-carvone and cuminyl alcohol, dihydrocarvone and l-carvone, estragole and l-carvone, d-carvone and l-carvone. Previous studies have also explored the potential for synergistic effects of essential oil components. Pavela (2015) assessed the acute toxicity of 30 aromatic compounds and their binary combinations against *Culex quinquefasciatus* larvae, and found 249 combinations showing significant synergistic effect. The mixture of limonene and transanethole caused the highest mortality, and l-carvone had a synergistic effect with 24 out of the 30 tested compounds. In addition, a study testing binary combinations of 6 monoterpenoids against *Musca domestica* found that p-cymene mixed with γ -terpinene, carvacrol and 1,8-cineole resulted in the most significant synergistic effect (Pavela, 2008). Our study confirmed that l-carvone, with low toxicity, created a synergistic effect with 7 monoterpenoids and an antagonistic effect with 1 monoterpenoid. Comparatively, 2-ethylimidazole, with high toxicity, was found to be antagonized with 5 monoterpenoids and synergized with 2 monoterpenoids.

Inhibition of AChE activity may be a mechanism for causing insect death. Previous studies have shown that

the oil extract of *Acalypha wilkesiana* inhibited AChE activity in adult *Callosobruchus maculatus* (Oni *et al.*, 2019), estragole inhibited AChE activity in *Tribolium castaneum* (Olmedo *et al.*, 2015), and cuminaldehyde, limonene and 1,8-cineole inhibited AChE activity in *S. oryzae* (Abdelgaleil *et al.*, 2009). Our study confirmed that estragole, cuminaldehyde, and 1,8-cineole inhibited AChE activity in *P. sinica*, although their insecticidal activities against *P. sinica* was weak. This is similar to the research reported by Kim *et al.* (2013), in which α -pinene showed the highest inhibition rate (97.36%) of AChE activity in *S. oryzae* but had low toxicity. We found that 2-ethylimidazole and cuminyl alcohol had the best toxicity to *P. sinica*, but had almost no effect on AChE activity *in vitro*, implying that AChE was not the target for 2-ethylimidazole or cuminyl alcohol. Furthermore, some essential oils also have inhibitory effects on insect GST activity. For instance, the essential oils of *Acalypha wilkesiana* significantly reduced GST activity in *Callosobruchus maculatus* (Oni *et al.*, 2019). The geraniol, linalool, citral, and 3-carene cause a significant reduction of GST activity in *Sitophilus zeamais* and *Callosobruchus maculatus* (Oyedeji *et al.*, 2020). Our results showed that 12 monoterpenoids tested had inhibitory effects on GST activity in *P. sinica*.

The synergistic effect of synergists is typically attributed to the inhibition of detoxification enzymes (Churcher *et al.*, 2016; Shen *et al.*, 2016). One substance's ability to inhibit GST activity may protect other toxins from degradation by GST (Metcalf, 1967). Degradation of toxins through multiple detoxification pathways in insects may decrease the antagonism of binary mixtures (Bernard and Philogene, 1993; Ishaaya, 1993). Piperonyl butoxide, a well-known inhibitor of cytochrome P450 monooxygenases and esterases, has a synergistic effect on chlorpyrifos, methomyl, acetamiprid, and spirotetramat by inhibiting the activity of cytochrome P450 monooxygenase (Ullah *et al.*, 2017). In this study, it seemed that the higher the GST inhibitory activity of the monoterpenoid, the fewer antagonistic binary mixtures it created. However, the synergistic or antagonistic mechanisms of these monoterpenoids still require further study.

CONCLUSION

Two monoterpenoids, 2-ethylimidazole and cuminyl alcohol, showed high toxicity against *P. sinica*. L-carvone was identified as the best synergist, and the maximal synergic binary mixtures were: l-carvone and dihydrocarvone, l-carvone and cuminaldehyde, l-carvone and cuminyl alcohol, l-carvone and d-carvone, l-carvone and estragole. These binary mixtures may have the potential to be used as effective control agents against *P.*

sinica. The findings of this study provide valuable insights into potential control strategies for prevention and control of *P. sinica*.

Funding

This work was supported by the National Natural Science Foundation of China (Grant No.31360182).

IRB statement

Not applicable, the study did not involve humans or animals.

Ethical statement

Not applicable.

Statement of conflict of interest

The authors have declared no conflict of interest.

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