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Research Article

Insight into the synergistic action of plant volatiles as biofumigants against stored product pests, *Sitophilus oryzae* (L.), *Tribolium castaneum* (H.), and *Callosobruchus maculatus* (F.), adults

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ABSTRACT: Plant volatiles *viz.*, Phytochemicals (PCs) and Essential Oils (EOs) are considered an eco-friendly alternative to the highly toxic conventional gaseous fumigants. Fumigant activity of two EOs (lemon and peppermint), two PCs (linalool and piperitone) and their binary combinations were tested at 10-200 μL/L air concentration against *Sitophilus oryzae* (L.), *Tribolium castaneum* (H.), and *Callosobruchus maculatus* (F.) adults. The Fumigant Toxicity Index (FTI) was estimated with phosphine as standard. All individual treatments were toxic against the examined insect pests. However, pipertione was identified as the most active and linalool was considered the least active fumigant. Linear regression of mortality and treatment concentration revealed a significant positive correlation for *S. oryzae* ($r^2 < 0.81$) and *T. castaneum* ($r^2 < 0.78$) and not for *C. maculatus* ($r^2 < 0.66$). Of the 6 binary combinations, 3, 4, and 1 combinations were synergistic against *S. oryzae, T. castaneum,* and *C. maculatus,* respectively. The peppermint plus piperitone combination was recorded with the highest synergy ratio 5.2 against *S. oryzae*. Further, FTI was higher for synergistic combinations than their corresponding components. Thereby, the present study suggests that synergistic binary combinations are more potent than individual treatment in controlling stored-product insect pests.

KEYWORDS: Binary combination, essential oil, phytochemical, stored-product pests, synergism

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INTRODUCTION

Insects are a major threat to food security causing severe qualitative and quantitative damages leading to food loss during post-harvest storage. The Rice weevil (*Sitophilus oryzae* (L.); Coleoptera: Curculionidae), Red flour beetle (*Tribolium castaneum* (H.); Coleoptera: Tenebrionidae), and Pulse beetle (*Callosobruchus maculatus* (F.); Coleoptera: Bruchidae) are most common insect pests infesting food grains and products (Rees, 2004). Gaseous fumigation is the most efficient way to control insect infestation in stored food commodities. Globally, phosphine fumigant has been potentially used during the past few decades. Another fumigant, methyl bromide has been phased out due to its ozone-depleting nature, and currently in India, it is under high restriction in use, especially for quarantine and pre-shipment purposes (CIBRC, 2022). Due to the sole and prolonged use of phosphine for bulk grain fumigation, many stored-product insect pests have developed resistance leading to control failures (Nayak *et al.*, 2020; Manivannan, 2015). As an alternative to conventional fumigants, plant volatiles, *i.e.*, PCs and EOs are being considered as potential sources for developing bio-fumigants with promising insecticidal activities and they are generally considered as environmentally safe (Hubert *et al.*, 2008). Especially, the majority of edible plants based EOs and PCs are recognized as Generally Regarded As Safe (GRAS) by the USFDA (https://www.ecfr.gov/current/title-21/chapter-I/subchapter-B/part-182).

In general, EOs contain terpenes and terpenoids as rich components with high vapour pressure which makes it a desirable attribute for fumigant toxicity in insect pests. Consequently, a large number of EOs and PCs have been screened for fumigant, contact, and repellent activities against a variety of stored-product insect pests (Rajendran & Sriranjini, 2008). Furthermore, few studies explore the

synergistic interaction of PCs and/or EOs against insect pests. Recently, studies on the synergistic behaviour of EOs and their components (Hategekimana & Erler, 2020), PCs (Liu *et al.*, 2019; Kanda *et al.*, 2017), crude extracts (Ileke *et al.*, 2016), and diatomaceous earth admixed with insecticides (Saeed *et al.*, 2022) against various stored-product insect pests have been reported. In our recent studies, we have examined the residual characteristics of PCs in fumigated grains (Vendan *et al.*, 2017), the persistence and ingestion characteristics of PCs in *S. oryzae* (Sahu *et al.*, 2021a), the impact of PCs on cuticular semiochemical secretions in *T. castaneum* (Sahu *et al.*, 2023), enhancement of fumigant activities of PCs using ozone as carrier gas (Sahu *et al.*, 2021b) and nanoemulsions (Ibrahim *et al.*, 2023) against insect pests, chitin characteristics against the PCs in insects (Gawali *et al.*, 2024) and influence of physiochemical properties of PCs on fumigant action in insect pests (Lokesh *et al.*, 2024). However, as per literature data, no plant-based fumigants are commercially available for stored-product insect pest management. Further, the synergistic combination of plant volatiles may facilitate their potential application in post-harvest grain storage management and protection from insect pest infestations. Therefore, this present study aimed at evaluating the synergistic interactions and fumigant activities of plant volatiles such as lemon EO, peppermint EO, linalool, and piperitone against pulse beetle (*C. maculatus*), grain beetle (*S. oryzae*), and flour beetle (*T. castaneum*) pests.

MATERIALS AND METHODS

Chemicals

EOs (lemon and peppermint) were purchased from Sigma-Aldrich (India), and PCs (linalool and piperitone) were procured from the Tokyo Chemical Industry (India).

Insect culture

The *S. oryzae*, *T. castaneum,* and *C. maculatus* were maintained regularly in the insectary unit at the study institute. Sub-cultures were prepared from the stock cultures, with approximately 500 adults fixed in the suitable culture media (whole wheat grain, wheat flour, and moong gram for *S. oryzae, T. castaneum,* and *C. maculatus,* respectively). Insect culture was maintained under controlled conditions (relative humidity 75 ± 5%, temperature 30 ± 2°C, light-dark period 13:11 h). After 7 days, adults were removed from the culture media using sieves. The cultures were regularly observed for the emergence of new insects. One-week-old *S. oryzae* and *T. castaneum,* and three days old *C. maculatus* adults were used for bioassays.

Fumigant toxicity of plant volatiles

To evaluate the fumigant activity of PCs and EOs, a method of Sahu *et al.* (2021a) was used with slight modifications. Ten adults of each species (*i.e.*, *S. oryzae*, *T. castaneum,* and *C. maculatus*) were separately introduced into a 50 mL glass vial. Whatman filter paper discs (1 cm^2) were loaded with EOs/PCs at four different concentrations (10 to 200 μL/L of air) and placed under the screw cap. Vials were screwed tight and sealed with parafilm to ensure airtight condition. Vials without any EO/PC treatment were used as a negative control. Phosphine gas was used as positive control as described by Vendan *et al.* (2017). Five replicates were maintained for each treatment concentration and control. Insect mortality was observed after 72 h of exposure period.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The chemical composition of EOs was analysed employing GC-MS (8890GC, 5977C GC/MSD, Agilent Technologies, USA) and HP-5MS column (30 m x 0.25 mm, 0.25 µm thickness, Agilent Technologies, USA). The conditions are as follows; oven temperatures initial 45°C (5 min), final 250°C (5 min), ramp rate 5°C/min; injection volume 1 μl (splitless mode); injection portal at 250°C; Helium as carrier gas (1 min solvent delay), scanning range 35-500 Da. Compounds were identified by mass spectra comparison in the NIST Tandem Mass Spectral Library (Licensed Version 3.0, 2023).

Synergistic interactions of binary combinations

Potential synergistic interactions among EOs and PCs were evaluated according to Tak *et al.* (2016). Binary combinations of individual EOs and PCs were prepared at equal proportions (1:1) and tested for their fumigant activity as described above. The synergistic relationship among the binary combinations was analysed according to Wadley's determination model (Wadley, 1945; 1967). Further, the Synergy Ratio (R) between the observed LC_{50} and the expected LC_{50} value was determined to predict the synergistic, additive, and antagonistic interactions.

Data processing and analysis

Insect mortality due to the fumigant effect of plant volatiles and their binary combinations was calculated using Abbott's formula for mortality correction (Abbott, 1925):

$$Corrected\ mortality\ (\%) = \frac{T-C}{100-C} \qquad \text{Eq. (1)}$$

where T is the mortality observed due to plant volatile treatment and C is the mortality observed in the negative control. To calculate the lethal concentrations (LC_{50} and LC_{90}), a probit analysis of mortality was performed (Finney, 1971). Linear regression analysis was performed to estimate the significance between insect mortality and varying concentrations of EOs and PC treatments.

Relative abundance (RA) of the compounds identified in EOs and PCs was calculated using the following formula:

$$RA = \frac{Peak\ area\ of\ compound}{Total\ area\ of\ compounds\ in\ EO/PC} * 100 \quad \text{Eq. (2)}$$

One-way ANOVA with Tukey's test was carried out to find the significance ($p<0.05$) between mortality and binary combination treatments for each pest species at every concentration. In addition, two-way ANOVA was conducted to estimate the significance between two factors (*i.e.*, treatments and species) with mortality as the dependent variable and concentration as a covariate. For ANOVA and Probit analysis, SPSS software (16.0 version) was used.

To assess the interaction of a binary combination of EOs and PCs, an expected LC_{50} value was calculated by Wadley's determination model (Wadley, 1945; 1967):

$$Expected\ LC_{50} = \frac{a+b}{\frac{a}{LC_{50(a)}}+\frac{b}{LC_{50(b)}}} \quad \text{Eq. (3)}$$

where, a is the concentration of component A in the mixture and $LC_{50(a)}$ is the observed LC_{50} value of the component. The R between the expected LC_{50} value and the observed LC_{50} value is determined as

$$R = \frac{Expected\ LC_{50}}{Observed\ LC_{50}} \quad \text{Eq. (4)}$$

where, the interaction is determined as synergistic ($R \geq 1.5$), additive ($1.5 > R \geq 0.5$), or antagonistic ($R < 0.5$).

The Fumigant Toxicity Index (FTI) of individual EOs and PCs and their binary combinations was determined using the following equation (Sun, 1950):

$$FTI = \frac{LC_{50}\ of\ the\ standard\ sample}{LC_{50}\ of\ the\ test\ sample} * 100 \quad \text{Eq. (5)}$$

where, phosphine was used as the standard.

RESULTS

Fumigation toxicity of individual PCs and EOs

All the tested PCs and EOs exerted fumigant toxicity against the stored-product beetle pests. The corrected mortality was calculated using Eq. (1). Linear regression revealed a positive correlation between observed mortality and the treatment concentration of EOs and PCs (Figure 1). There was no significant ($r^2 < 0.66$) increase in mortality of *C. maculatus* with the increase in treatment concentration. However, a significant increase in mortality with the increase in concentration was observed against *S. oryzae* ($r^2 > 0.81$) and *T. castaneum* ($r^2 > 0.78$). Mortality data indicates that *C. maculatus* was more susceptible than the other two species due to >50% mortalities at the lowest treatment concentration (10 μL/L air) of EOs and PCs. Significant differences between all treatments ($F = 12.47$; $df = 3$; $p<0.0001$) and the three insect species ($F = 95.74$; $df = 2$; $p<0.0001$), and for the treatment-insect interaction ($F = 5.89$; $df = 6$; $p<0.0001$) was shown by two-way ANOVA (Table 1). Probit analysis showed that all treatments (except linalool) were potent against *S. oryzae*, *T. castaneum,* and *C. maculatus* with LC_{50} values of < 100, < 160, and < 15 μL/L air, respectively (Table 2).

Composition of EOs and PCs

Figure 2 and Table 3 show the chemical profile of the tested EOs and PCs. A total of 18 compounds were detected in GC-MS analysis. The relative abundance of the detected compounds was calculated using Eq. (2). Limonene (92.27%) and isomenthone (47.20%) were detected as the major compounds in lemon and peppermint EO, respectively. The linalool and piperitone PCs were found with 99.20 and 98.55% purity.

Synergistic interaction of binary combinations

All the examined binary combinations exhibited fumigant toxicity against the insect pests. Complete mortality (100%) of *C. maculatus* was observed for all six binary combinations at 200 μL/L air (Figure 3). Whereas against *S. oryzae* and *T. castaneum*, four (except PEP+LIN and LIN+PIP) and five (except PEP+LIN) binary combinations showed 100% mortalities at 200 μL/L air, respectively. Probit analysis showed that all binary combinations were potent against *S. oryzae*, *T. castaneum* and *C. maculatus*, with LC_{50} values of < 140 < 105 and < 50 μL/L air, respectively (Table 4). Two-way ANOVA demonstrated that insect mortality differed significantly among insect pests ($F = 41.99$; $df = 2$; $p<0.0001$), among binary combinations ($F = 6.48$; $df = 5$; $p<0.0001$), and the combination-insect interaction ($F = 8.33$; $df = 10$; $p<0.0001$) (Table 5).

Based on Wadley's determination model and R ratio (*i.e.*, Eqs. 3 and 4, respectively), three, four, and one synergistic interactions were observed against *S. oryzae*, *T. castaneum,* and *C. maculatus*, respectively (Table 6). The binary combination PEP+PIP recorded the highest R value of 5.2 against *S. oryzae*, followed by LEM+LIN with an R value of 4.5 against *T. castaneum*.

Further, using Eq. (5), FTI values of the binary combinations with respect to phosphine (positive control) were calculated. Consequently, synergistic binary combinations were observed to have higher FTI values than their corresponding individual components (Figure 4). For example, the synergistic combination LEM+PEP against *C. maculatus* possessed higher FTI (60.44) than its components, lemon (25.07) and peppermint EO (28.93). Similarly, synergistic combinations PEP+PIP and LEM+LIN against *S. oryzae*, and *T. castaneum* had higher FTI than their corresponding components.

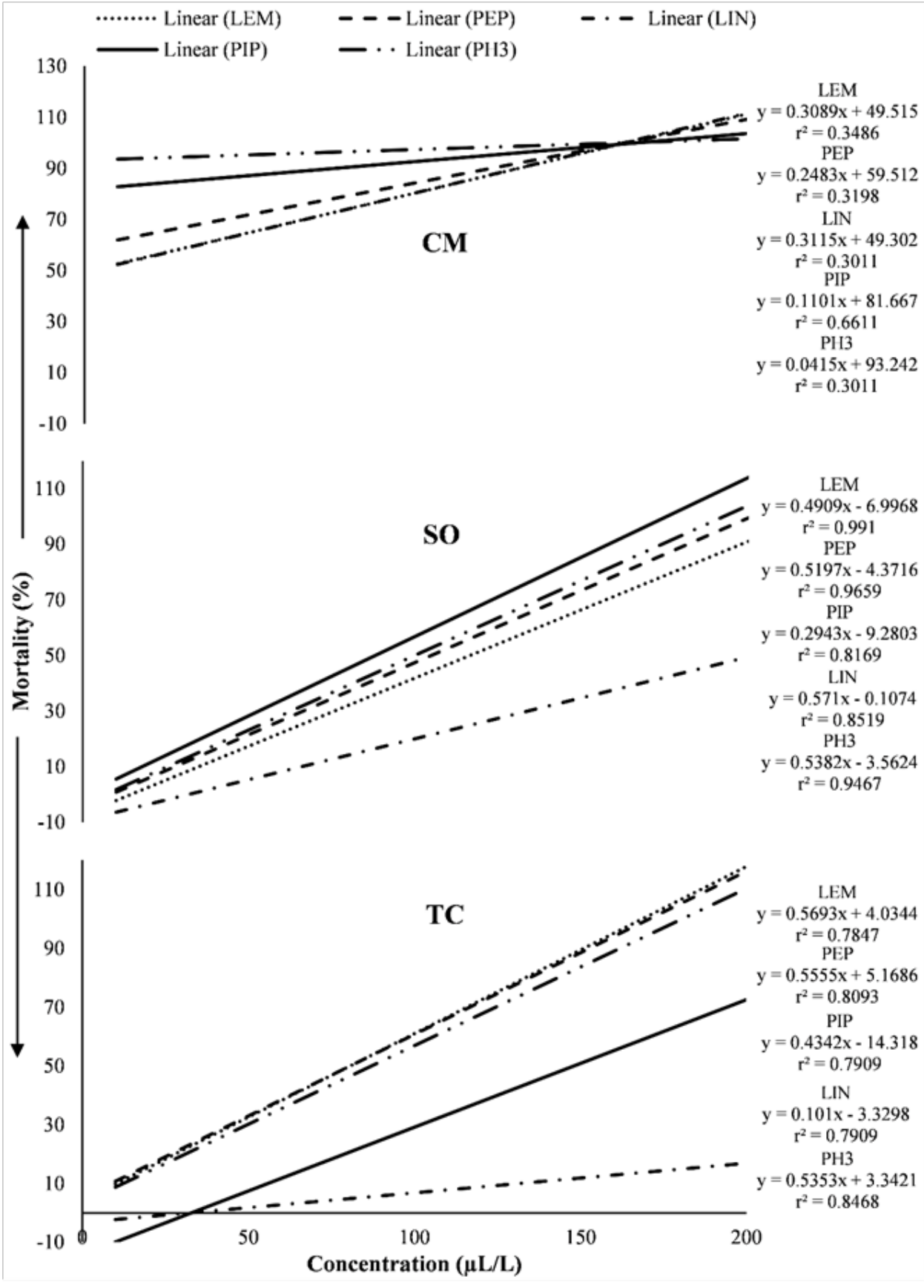


Figure 1. Linear regression showing the relationship between mortality of insect pests and fumigant concentrations. LEM - Lemon EO, PEP - Peppermint EO, LIN - Linalool, PIP - Piperitone, PH_3 - Phosphine, SO - *S. oryzae*, TC - *T. castaneum*, CM - *C. maculatus*.

Table 1. Two-way ANOVA results testing the fumigation toxicity of plant volatiles and insect pest species

Source	Type III Sum of Squares	df	Mean Square	F	p
Corrected Model	362958.53[a]	12	30246.54	48.35	<0.0001
Intercept	34132.42	1	34132.42	54.56	<0.0001
Concentration	197659.88	1	197659.88	315.98	<0.0001
Insect	119776.46	2	59888.23	95.74	<0.0001
Treatment	23406.15	3	7802.05	12.47	<0.0001
Insect × Treatment	22116.04	6	3686.01	5.89	<0.0001
Error	141998.87	227	625.55		
Total	1071925.00	240			
Corrected Total	504957.40	239			

[a]r Squared = 0.72 (Adjusted r Squared = 0.70), df = degrees of freedom

Table 2. Fumigant toxicity of essential oils and phytochemicals against insect pests

Treatment	LC_{50} (LL - UL)[a]	LC_{90} (LL - UL)[a]	Slope ± SE	χ^2 (df)
C. maculatus				
LEM*	14.32 (13.43 - 15.27)	20.50 (18.91 - 22.79)	8.22 ± 0.37	68.81 (18)
PEP*	12.41 (11.27 - 13.58)	21.03 (18.47 - 25.77)	5.59 ± 0.33	85.23 (18)
LIN*	14.21 (13.54 - 14.89)	18.08 (17.19 - 19.15)	12.25 ± 0.59	30.53 (18)
PIP*	5.15 (1.03 - 8.17)	20.32 (15.00 - 39.40)	2.15 ± 0.22	126.22 (18)
PH_3	3.59 (0.00 - 6.65)	10.68 (0.87 - 16.55)	2.71 ± 0.63	34.13 (18)
S. oryzae				
LEM*	97.87 (74.79 - 124.29)	239.35 (178.25 - 406.95)	3.30 ± 0.15	260.25 (18)
PEP*	89.19 (80.09 - 97.12)	170.19 (154.67 - 192.61)	4.57 ± 0.29	41.03 (18)
LIN*	192.27 (175.44 - 175.44)	345.25 (287.36 - 473.13)	5.04 ± 0.35	65.30 (18)
PIP*	69.10 (41.80 - 79.83)	103.77 (97.49 - 113.83)	7.23 ± 1.57	26.38 (18)
PH_3	82.62 (62.43 - 96.73)	150.44 (128.81 - 197.13)	4.92 ± 0.43	44.78 (18)
T. castaneum				
LEM*	34.63 (29.33 - 49.59)	51.46 (39.26 - 94.78)	7.45 ± 1.03	33.19 (18)
PEP*	41.06 (36.91 - 45.86)	77.76 (68.07 - 91.09)	4.62 ± 0.17	47.15 (18)
LIN*	426.57 (303.97 - 1157.76)	1169.46 (599.84 - 9051.57)	2.93 ± 0.43	49.22 (18)
PIP*	154.11 (140.78 - 168.09)	224.67 (201.97 - 263.01)	7.83 ± 0.38	118.97 (18)
PH_3	52.76 (39.33 - 68.95)	119.83 (88.87 - 180.42)	3.60 ± 0.17	88.70 (18)

LEM - Lemon EO, PEP - Peppermint EO, LIN - Linalool, PIP - Piperitone, PH_3 - Phosphine, LL - UL - Lower and Upper limit, [a] 95% confidence interval, LC_{50} expressed in μL/L, * Data from our previous study Lokesh *et al.* (2024)

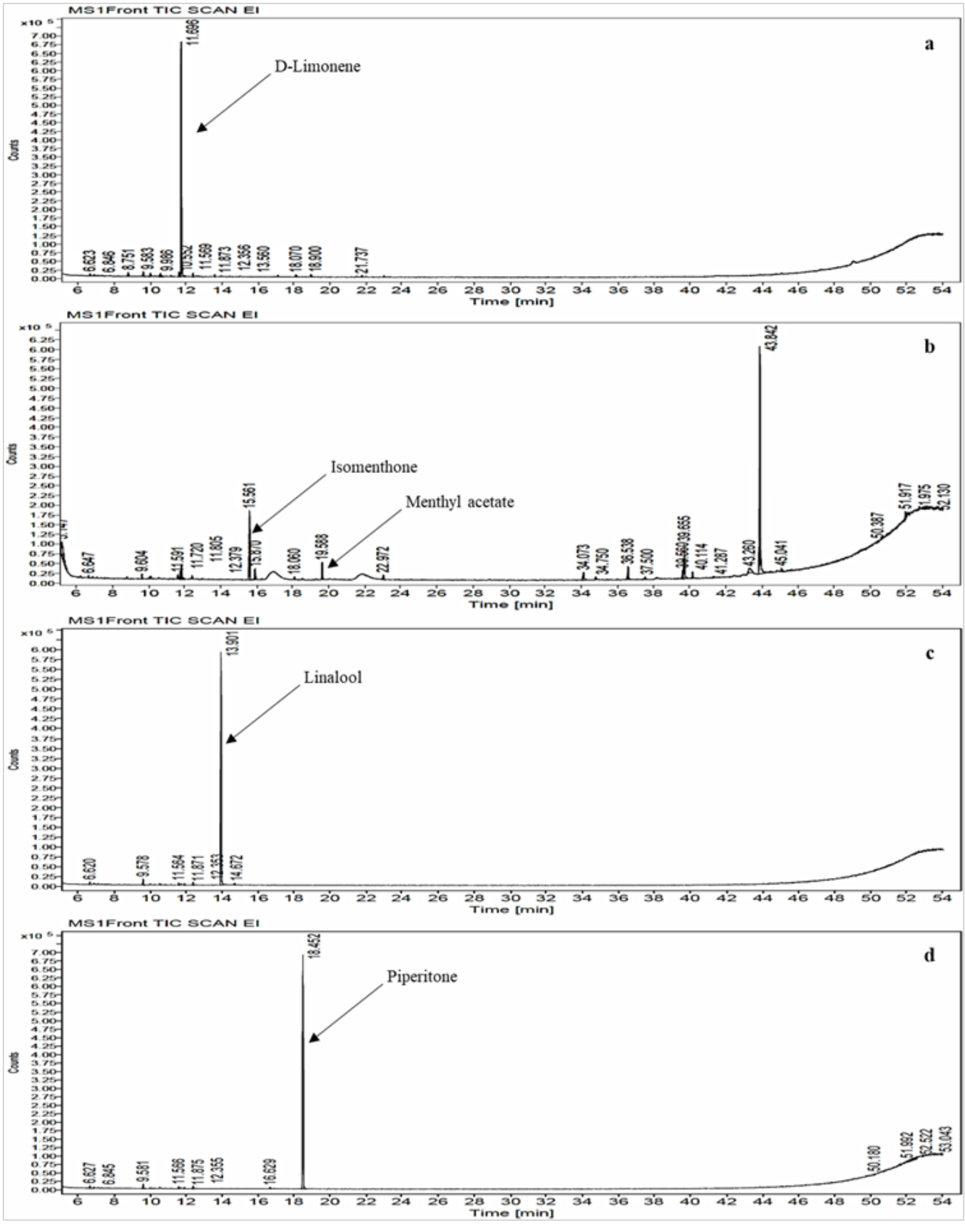


Figure 2. GC-MS chromatogram of plant volatiles. **(a)** Lemon EO, **(b)** Peppermint EO, **(c)** Linalool, **(d)** Piperitone.

Table 3. Chemical composition of plant volatiles

RT (min)	Compound	Relative abundance (%)			
		LEM	**PEP**	**LIN**	**PIP**
9.99	β-Terpinene	1.43	-	-	-
10.55	β-Pinene	3.02	-	-	-
11.70	D-Limonene	92.27	-	-	-
11.81	Eucalyptol	-	12.53	-	-
11.88	Decane	-	-	-	0.71
13.56	Terpinolene	0.90	-	-	-
13.90	Linalool	-	-	99.20	-
14.67	Plinol C	-	-	0.80	-
15.56	Isomenthone	-	47.20	-	-
15.87	L-Menthone	-	11.64	-	-
16.63	α-Terpineol	-	-	-	0.74
18.06	Pulegone	-	1.87	-	-
18.07	Neral	1.22	-	-	-
18.45	Piperitone	-	-	-	98.55
18.90	Geranial	1.16	-	-	-
19.59	Menthyl acetate	-	12.70	-	-
22.97	Caryophyllene	-	3.56	-	-
34.07	Methyl isohexade-canoate	-	10.51	-	-

RT - Retention time, LEM - Lemon EO, PEP - Peppermint EO, LIN - Linalool, PIP - Piperitone

DISCUSSION

Insect-plant interactions have led plants to evolve defensive strategies to protect themselves from herbivorous insects by producing many secondary metabolites (War *et al.*, 2012). Plants produce EOs as secondary metabolites (O'Bryan *et al.*, 2015). Numerous EOs have been studied for their fumigant activity against various stored-product insect pests. The present study demonstrated the fumigant toxicity potentiality of lemon EO, peppermint EO, linalool, and piperitone against the stored-product insect pests. As a result, peppermint EO, lemon EO, and piperitone were identified as potent biofumigants. Recently, peppermint EO was reported as an effective fumigant and contact toxicant against *T. castaneum* and *S. oryzae* (Mackled *et al.*, 2019). A study by Nagar *et al.* (2012), showed a profound fumigant effect of peppermint volatiles against the development of all stages of *C. maculatus* including fecundity. Moravvej and Abbar (2008) studied the fumigant toxicity of four *Citrus* spp. EOs and concluded that all EOs were effective against *C. maculatus* at varying degrees. Similarly, Abdelgaleil *et al.* (2016) reported lemon EO to be the second most effective fumigant among 20 EOs tested against *S. oryzae*. Previously, piperitone was identified as a major compound of *Cymbopogon schoenanthus* EO and was reported as a potent fumigant against *T. castaneum* and *C. maculatus* (Ketoh *et al.* 2006; Bossou *et al.*, 2015). When compared to *S. oryzae* and *T. castaneum*, *C. maculatus* was considered as most susceptible to all the treatments including the least active linalool. Similarly, Davoudi *et al.* (2011) reported linalool to be effective against *C. maculatus*. Eljazi *et al.* (2017) reported that linalool was an effective PC against *T. castaneum* and *S. oryzae*.

In the present study, GC-MS analysis revealed that limonene was found as a major component with 92.27% in lemon EO and five different components (isomenthone, eucalyptol, L-menthone, menthyl acetate and methyl isohexadecanoate with >10%) were found as major components in peppermint EO, which represents as principal insecticidal bioactives in EOs. The highly pure linalool (99.20%) and piperitone (98.55%) exhibited insecticidal activities against the examined insect pests. Viuda-Martos *et al.* (2009) studied the EO composition of four citrus

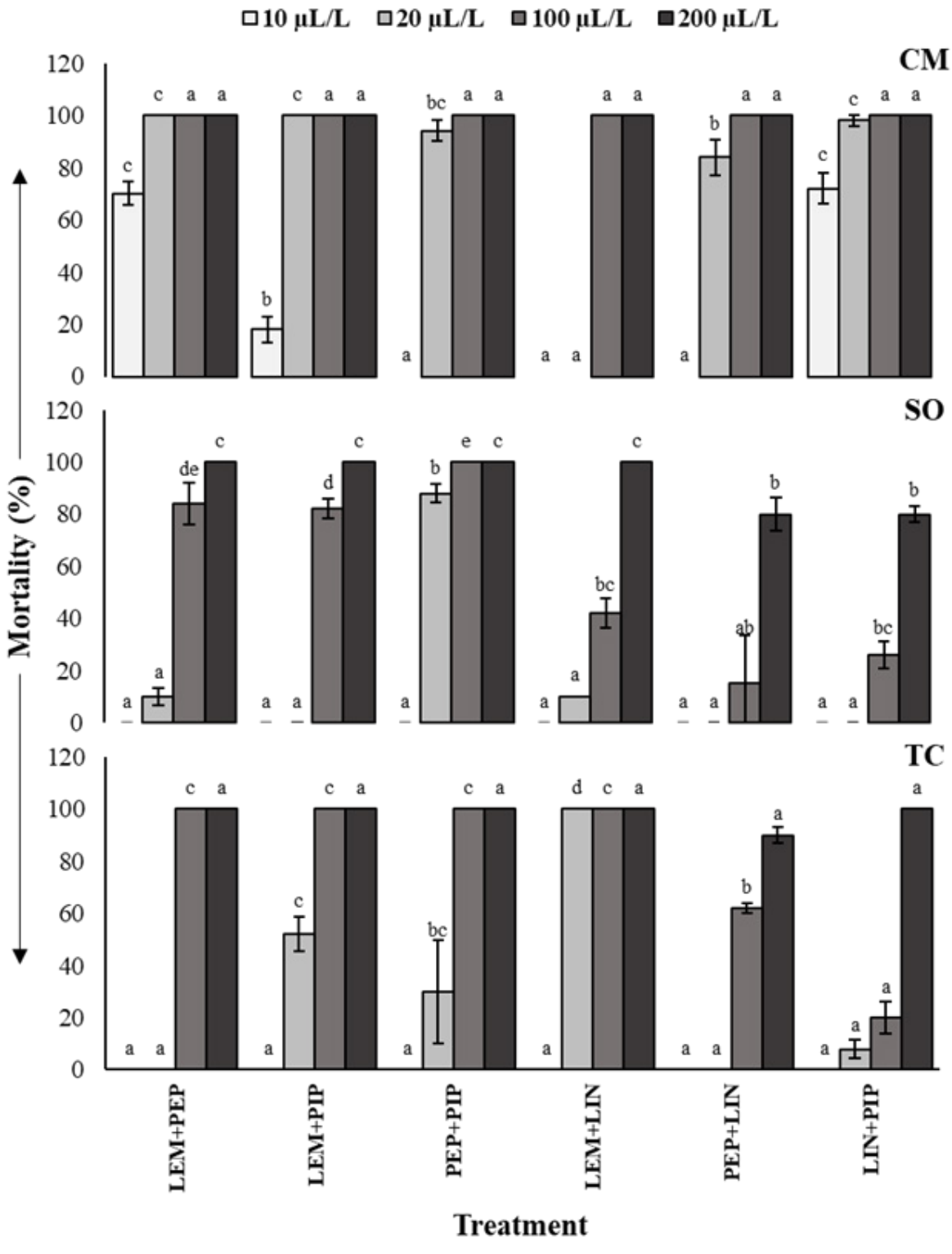


Figure 3. Mortality of three major stored-product insect pests with the effect of binary combinations of plant volatiles at various concentrations. LEM - Lemon EO, PEP - Peppermint EO, LIN - Linalool, PIP - Piperitone, SO - *S. oryzae*, TC - *T. castaneum*, CM - *C. maculatus*. Bars represent a mean of 5 replicates with standard error. Mean values within a treatment concentration followed by different letters are significantly different ($P < 0.05$) of mortality as determined by Tukey's test.

Table 4. Fumigant toxicity of binary combinations against three stored-product insect pests

Treatment	LC_{50} (LL - UL)[a]	LC_{90} (LL - UL)[a]	Slope ± SE	χ^2 (df)
C. maculatus				
LEM+PEP	5.94 (3.13 - 7.69)	15.98 (13.63 - 21.69)	2.98 ± 0.36	58.78 (18)
LEM+PIP	12.66 (11.22 - 14.19)	21.50 (18.38 - 28.33)	5.57 ± 0.33	127.67 (18)
PEP+PIP	15.10 (14.18 - 15.95)	19.26 (18.22 - 20.53)	12.12 ± 0.58	57.93 (18)
LEM+LIN	44.79 (36.28 - 55.20)	61.16 (49.85 - 78.13)	9.47 ± 1.22	0.47 (18)
PEP+LIN	16.15 (14.95 - 17.39)	23.32 (21.23 - 26.69)	8.03 ± 0.41	90.30 (18)
LIN+PIP	7.24 (5.14 - 8.55)	14.43 (12.80 - 17.67)	4.28 ± 0.43	63.81 (18)
S. oryzae				
LEM+PEP	48.83 (39.89 - 59.41)	110.22 (88.14 - 147.70)	3.62 ± 0.12	160.88 (18)
LEM+PIP	77.12 (60.69 - 84.43)	111.95 (106.93 -124.16)	7.92 ± 1.88	18.88 (18)
PEP+PIP	15.74 (14.73 - 16.76)	22.70 (20.92 - 25.33)	8.07 ± 0.40	67.47 (18)
LEM+LIN	106.84 (74.79 - 138.62)	252.67 (183.45 - 553.30)	3.43 ± 0.19	337.52 (18)
PEP+LIN	77.86 (60.45 - 99.00)	217.14 (160.46 - 344.40)	2.87 ± 0.11	221.83 (18)
LIN+PIP	135.03 (126.40 - 143.98)	245.56 (220.75 - 284.26)	4.93 ± 0.29	39.54 (18)
T. castaneum				
LEM+PEP	44.79 (36.28 - 55.20)	61.16 (49.86 - 78.13)	9.47 ± 1.22	0.47 (18)
LEM+PIP	19.80 (18.83 - 20.98)	29.03 (26.32 - 33.87)	7.72 ± 0.60	40.89 (18)
PEP+PIP	22.52[b]	30.17[b]	10.09 ± 2.96	380.01 (18)
LEM+LIN	14.35 (13.71 - 15.02)	18.26 (17.37 - 19.35)	12.25 ± 0.56	34.19 (18)
PEP+LIN	88.13 (79.62 - 96.10)	186.58 (168.67 - 211.75)	3.93 ± 0.21	38.10 (18)
LIN+PIP	100.22 (65.61 - 154.90)	272.37 (171.41 - 832.21)	2.95 ± 0.12	570.43 (18)

LEM - Lemon EO, PEP - Peppermint EO, LIN - Linalool, PIP - Piperitone, LL - UL - Lower and Upper limit, [a] 95% confidence interval, [b] LL and UL was not computed due to variation

LC_{50} expressed in μL/L

Table 5. Two-way ANOVA results testing the fumigation toxicity of binary combinations and insect species

Source	Type III Sum of Squares	df	Mean Square	F	*P*
Corrected Model	506886.82[a]	18	28160.38	40.16	<0.0001
Intercept	68422.79	1	68422.79	97.58	<0.0001
Concentration	366876.82	1	366876.82	523.23	<0.0001
Insect	58881.67	2	29440.83	41.99	<0.0001
Treatment	22716.67	5	4543.33	6.48	<0.0001
Insect × Treatment	58411.67	10	5841.17	8.33	<0.0001
Error	239103.18	341	701.18		
Total	1828400.00	360			
Corrected Total	745990.00	359			

[a]r Squared = 0.68 (Adjusted r Squared = 0.66), df = degrees of freedom

Table 6. Synergistic interactions of binary combinations against three stored-product insects by fumigant toxicity

Insect species	Treatment	Observed LC_{50} [a]	Expected LC_{50} [a]	*R*	Interaction
CM	LEM+PEP	5.94	13.30	2.2	Synergistic
	LEM+PIP	12.66	7.58	0.6	Additive
	PEP+PIP	15.10	7.28	0.5	Additive
	LEM+LIN	44.79	14.26	0.3	Antagonistic
	PEP+LIN	16.15	13.25	0.8	Additive
	LIN+PIP	7.24	7.56	1.0	Additive
SO	LEM+PEP	48.83	93.33	1.9	Synergistic
	LEM+PIP	77.12	81.01	1.1	Additive
	PEP+PIP	15.74	81.01	5.2	Synergistic
	LEM+LIN	106.84	129.71	1.2	Additive
	PEP+LIN	77.86	121.85	1.6	Synergistic
	LIN+PIP	135.03	101.66	0.8	Additive
TC	LEM+PEP	44.79	37.57	0.8	Additive
	LEM+PIP	19.8	56.55	2.7	Synergistic
	PEP+PIP	22.52	64.84	2.9	Synergistic
	LEM+LIN	14.35	64.06	4.5	Synergistic
	PEP+LIN	88.13	74.91	0.9	Additive
	LIN+PIP	100.22	226.42	2.3	Synergistic

LEM - Lemon EO, PEP - Peppermint EO, LIN - Linalool, PIP - Piperitone, SO - *S. oryzae*, TC - *T. castaneum*, CM - *C. maculatus*, [a] 95% confidence interval, Synergy ratio (*R*), interaction was determined as synergistic ($R \geq 1.5$), additive ($1.5 > R \geq 0.5$), or antagonistic ($R < 0.5$), LC_{50} expressed in μL/L

fruit peels (including lemon) and reported limonene as the most abundant compound which is in accordance with the present study. The composition of peppermint EO, however, depends on the stage of the leaf development as noted by Gershenzon *et al.* (2000). They observed that the youngest leaves of peppermint were majorly composed of limonene and menthone, with the development of limonene content reduced and menthone increased, at later stages menthone declined as menthol became the dominant constituent.

The insecticidal activity exerted by a mixture of two or more components could be higher or lower than their components leading to either synergism, additive, or antagonistic interaction. In the present study, the synergistic interaction of binary combinations was determined with the aid of Wadley's model. This model works on the assumption that the activity of the components in a mixture has a similar mode of action, where one component can substitute another (Kosman & Cohen, 1996). Tak *et al.* (2016) used

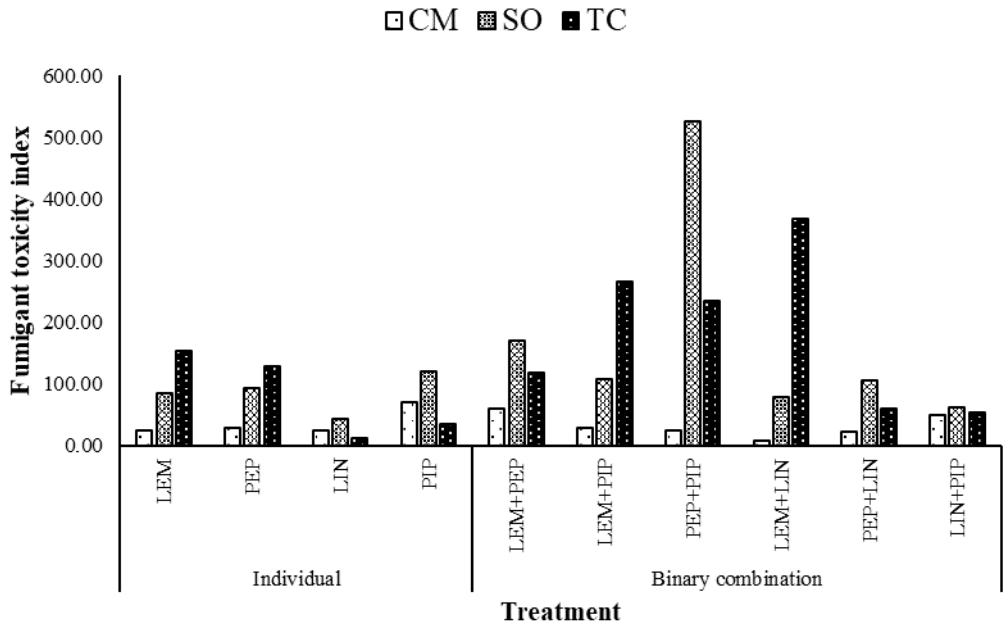


Figure 4. Fumigant toxicity index of individual plant volatiles and their binary combinations against the three major stored-product insect pests. LEM - Lemon EO, PEP - Peppermint EO, LIN - Linalool, PIP - Piperitone, SO - *S. oryzae*, TC - *T. castaneum*, CM - *C. maculatus*.

Wadley's model to analyse the synergistic interaction among the major monoterpenes (camphene, camphor, α-pinene, and 1,8-cineol) of rosemary EO against *Trichoplusia ni*. Similarly, in the present study, Wadley's model was used for understanding the synergistic interactions among the PCs and EOs containing PCs mainly composed of monoterpenes (d-limonene, isomenhone, linalool, L-menthone, piperitone, α-terpineol, etc,). Further, to the best of our knowledge, no reports are available on the fumigant activity of the binary combinations based on lemon EO, peppermint EO, linalool, and piperitone against the three examined stored-product insect pests. In the present study, the LEM+PEP binary combination was synergistic against the *C. maculatus* and *S. oryzae*. Supportively, Vendan *et al.* (2017) reported higher fumigant toxicity for the lemon and peppermint EO mixture (LC_{50} = 24.6 μL/L) compared to their components against *S. oryzae* (233.4 and 45.2 μL/L for lemon and peppermint EO, respectively). A recent study by Hategekimana and Erler (2020) showed the synergistic action of peppermint EO with anise EO against *S. oryzae*. Similarly, the synergistic behaviour of limonene (a major compound of lemon EO) and linalool was reported against *S. oryzae* in a fumigant assay (Liu *et al.*, 2019). Interestingly, the other binary combinations examined in the current study had varying effects on the studied insect pests. Similar findings have been reported by several studies. For instance, topical application of α- and β-pinene blends at different ratios behaved synergistically against *T. castaneum* while antagonistic (blending ratio, α-pinene: β-pinene = 6:1) and additive (blending ratio, α-pinene: β-pinene = 8:1) behaviours were observed against *Liposcelis bostrychophila* (Feng *et al.*, 2023). Additionally, different interactions were recorded between two species of a genus, *i.e.*, a mixture of wild marigold and peppermint EO was synergistic against *C. chinensis* and no synergism was observed against *C. maculatus* (Jayaram *et al.*, 2022). In another study, contact toxicity of limonene and linalool was studied where synergism was observed against the adult stage of *T. castaneum* while antagonism was seen against the larval stage of *T. castaneum* (Liang *et al.*, 2022). The authors opined that different life stages had varying levels of sensitivity to chemicals. Moreover, Qi *et al.* (2023) pointed out that the blending ratio of components in a mixture had different responses on an insect species. They observed the synergistic, and additive effect of bornyl acetate and benzyl benzoate mixture at 1:4 and 1:1 ratio, respectively against *T. castaneum*. From the previous reports and the present study findings, it is predicted that the interaction exhibited by a combination is not influenced by a single factor and it might be due to multiple factors such as the blending ratio and composition of the components as well as the species, life stage, age, and susceptibility/sensitivity of insect pest. Further, the FTI of binary combinations especially that of synergistic combinations could be observed to be higher than their components. This in turn implies that synergistic binary combinations are more effective than phosphine which could be attributed to the difference in modes of action between phosphine and plant volatiles in controlling insect pests. The prime difference is the mode of entry where phosphine

enters through inhalation while plant volatiles enter through penetration *via* the cuticle and respiratory system due to their lipophilic nature in addition to inhalation (Hubert *et al.*, 2008; Sahu *et al.*, 2021a). Phosphine is known to cause toxicity mainly by inducing oxidative stress, disrupting mitochondrial respiration (Nicotinamide Adenine Dinucleotide (NADH) and Adenosyl Triphosphate (ATP) deficiency), and causing neurotoxicity (acetylcholine esterase (AchE) inhibition) (Nath *et al.*, 2011). On the other hand, plant volatiles, specifically monoterpenes that constitute EOs, are believed to cause neurotoxicity by inhibiting or interfering with AchE, octopaminergic system and GABA-gated chlorine channels leading to insect paralysis and ultimately to death (Priestley *et al.*, 2006; Enan, 2005; Chaubey, 2016; Rodríguez *et al.*, 2022). In addition, plant volatiles inhibit ATPase, glutathione-S-transferase, P_{450} cytochromes, amylase, etc., impairing the energy production and detoxification processes, leading to insect knockdown effect and death (Karabörklü & Ayvaz, 2023). Moreover, Sahu *et al.* (2021a) observed that PCs (diallyl disulfide and eucalyptol) caused the rupture of scales and pits and setae malformation on the elytral surface in *S. oryzae*. Thus, unlike phosphine, plant volatiles have the added benefit of causing physical damage to insect cuticle. Further studies need to be conducted to explore the potential biofumigant with synergistic potentialities for the effective control of stored-product insects including *C. maculatus, S. oryzae,* and *T. castaneum*.

CONCLUSION

The present study explored the various interactions exhibited by binary combinations of EOs and PCs and their potential application in controlling stored-product insect pests. The results indicate that binary combinations with synergistic interactions were more effective than individual treatments. The binary combinations *viz.*, PEP+PIP, LEM+LIN, and LEM+PEP have been shown to have strong synergistic behaviour against *S. oryzae, T. castaneum,* and *C. maculatus,* respectively. The study further concludes that multiple factors may govern the interaction exerted by a mixture such as the blending ratio, composition of components along with species, life stage, age, and sensitivity of insect pests.

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