SUPPLEMENTARY MATERIAL

Fumigant and contact toxicities of individual and additive combinations of biorational-essential oils for control of rice weevil (*Sitophilus oryzae)*

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Abstract

Fumigant and contact activity of individual and combination of EOs (cumin seed and black pepper oil) was conducted against rice weevil (*Sitophilus oryzae*). Cumin seed and black pepper oil individually exhibited 100% insecticidal activity (Contact and Fumigant) for *S. oryzae* at 24 and 168 hours of exposure in absence and presence of food respectively. Overall, differential insect mortality rates were observed with individual EOs, however cumin seed EO showed higher insecticidal activity in both fumigant and contact conditions than black pepper EO. Binary mixtures of cumin seed and black pepper oil at 60:40 and 80:20 ratios showed higher toxicity than individual EOs against *S. oryzae* and further, additive interaction of binary combinations of EOs resulted in complete insecticidal activity of *S. oryzae*. Thus, we conclude that binary mixtures of cumin seed and black pepper EOs might be a promising prophylactic treatment solution for control of *S. oryzae* to prevent stored grain infestation.

Key words: Cumin seed oil, Black pepper oil, *Sitophilus oryzae*, binary combination, essential oil

1. Experimental

1.1 Insect rearing

The culturing of *Sitophilus oryzae* (L.) was done at the insectary unit of Food Protectants and Infestation Control Department, CSIR- Central Food Technological Research Institute, Mysuru. The insects were maintained on whole wheat grains at conditions of 75 ± 5% r.h., 13h: 11h (L:D) and controlled temperature (30 ± 2°C). The adults of *S. oryzae* from stock culture was used for sub- culturing in a 500 ml glass jar containing conditioned grains at 60°C for 5 mins to obtain the first generation (F1) and after a week the adults were removed from the jar. At regular observance, the emerged one month old insects of mixed sex were used for the bioassays.

1.2 Source of chemicals

The essential oils of Cumin seed oil (batch number SO-114/2021) and Black pepper oil (batch number SO-112/2021) were procured from Nishant Aromas, Mumbai, India. The solvents like n-hexane (HPLC grade 99.9% purity) were purchased from Merck chemicals.

1.3 *GC mass spectrometry (GC-MS) analysis*

GC-MS analysis of essential oils was carried out using PerkinElmer AutoSystemXL gas chromatograph equipped with quadrupole mass spectrometer (Model: Turbo-mass Gold, Watham, MA, USA) fitted with RTx-5 Crossbond 5% diphenyl/ 95% dimethyl polysiloxane capillary column (30 m x 0.25 mm x 0.25 µm film thickness; Restek, USA). Helium was used as carrier gas and GC-MS conditions used were with initial temperature of 40 °C for 2 min. Ramp was set to 5 °C/min to 260 °C/min with an injection volume of 1µL. 10 µL of sample was diluted in 990 µL of n-Hexane. Quantification and identification of compounds were based on peak area percentage of chromatograms. Major compounds present in essential oil were identified based on their fragmentation pattern referring to NIST 2011 Mass spectral library.

1.4 Insect bioassay

1.4.1 Fumigant activity of individual oils

The EO (cumin seed and black pepper oil) were evaluated for its insecticidal properties against *S. oryzae* adults under without and with food (whole wheat grains) conditions. The treatment volumes of EO include 5, 10, 15, 20 and 25 µL and their corresponding concentrations were166.7 µL/L air, 333.4 µL/L air, 500 µL/L air, 666.7 µL/L air and 833.4 µL/L air

respectively. In the without food conditions, the EOs were directly applied on L-shaped filter paper (Whatman No 1) of size 5 x 1 cm (Length x Breadth) and air-dried for 2 mins, after which they were suspended to the cap of a 30 ml glass vial for each treatment. A control without any EO treatment was prepared. Both the test and control treatments received 10 insects per vial. Parafilm was used to ensure an air tight condition in all the vials. For the fumigant activity in with food condition, the same experimental setup was followed including the use of 10 grams of whole wheat grains as food in the test and control vials. Each test and control were replicated three times. Observation of insect survival was recorded at 4 hr, 8 hr, 12 hr and 24 hr. The insects were considered dead if having no visible motility of any body part (Appendages, Antennae etc.)

1.4.2 Fumigant activity of binary combination

The fumigant activity of binary combinations of cumin seed and black pepper oil *viz.* 0:100, 20:80, 40:60, 60:40, 80:20 and 100:0 ratios were evaluated against *S. oryzae* adults under with and without food (Whole wheat grains) condition where a constant treatment quantity of 25 µL was used for each 30 mL tube. The EO's were directly applied on L-shaped filter paper (Whatman No 1) of size 5 x 1 cm (Length x Breadth) and dried for 2 mins, after which they were air-suspended on to the cap of a 30 ml glass vial for each with and without food treatments respectively. A control without any EO treatment was prepared. Both the test and control treatments received 10 insects per vial. Parafilm were used to ensure an air tight condition in all the vials. Each test and control were replicated three times Observation of insect survival was recorded at 4 hr, 8 hr, 12 hr and 24 hr. The insects were considered dead if having specific body curvature and no visible motility of any body part (Appendages, Antennae etc.) The effect of binary combinations was evaluated according to method of Trisyono and Whalon, 1999. Expected mortality was estimated as follows:

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

Where E is the expected mortality rate, O_a and O_b are observed mortalities of first and second compounds used in binary combination respectively. Determination of synergism, antagonism or additive effect of compounds was analysed by Chi-squared (X^2) comparison:

$$X^2 = \frac{(Om-E)^2}{E}$$

where E is the expected mortalities of combinations of *O. gratissimum* oil and carvacrol and Om is observed mortality. The tabular X^2 value with $df = 1$ and $\alpha = 0.05$ is 3.84, if X^2 value

>3.84 the binary combination may show either synergistic or antagonistic action. A greater value for observed mortality (Om) than expected mortality (E) is considered synergistic action and if observed mortality is smaller than expected mortality then antagonistic. If X^2 value< 3.84 it was concluded that the effect was additive.

1.4.3. Contact activity of individual oils

The EO (Cumin seed and black pepper oil) were evaluated for its insecticidal properties as a contact insecticide against *S. oryzae* adults under without and with food (Whole wheat grains) condition where the treatment volumes of EO used per area of 50.28 cm^2 of filter paper were 5, 10, 15, 20 and 25 µL hence corresponding to concentrations *viz.* 0.09 µL/cm^2,0.19 µL/cm^2, 0.29 µL/cm^2, 0.39 µL/cm^2 and 0.49 µL/cm^2 respectively. In the without food conditions, the EO were uniformly applied on filter paper (Whatman No 1) of diameter 80 mm (Area ~50.28 cm^2) and dried for 2 mins, after which they were kept in 100 mm petri plates for each treatment. For the contact activity in with food condition, the same experimental setup was followed where in the use of 10 grams of whole wheat grains was included as food in the test and control vials A control without any EO treatment was prepared. Both the test and control treatments received 10 insects per plate. Parafilm were used to ensure an air tight condition in all the petri plates. Each test and control were replicated three times. Observation of insect survival was recorded at 4 hr, 8 hr, 12 hr and 24 hr. The insects were considered dead if having specific body curvature and no visible motility of any body part (Appendages, Antennae etc.)

1.4.4. Contact activity of Binary combination

The contact activity of binary combination of Cumin seed and black pepper oil *viz.* 0:100, 20:80, 40:60, 60:40, 80:20 and 100:0 ratios were evaluated for its insecticidal properties against *S. oryzae* adults under with and without food (Whole wheat grains) condition where a constant treatment quantity of 25 µL was used for each plate (Diameter ~100 mm). The EO's were directly applied on a filter paper (Whatman No. 1) 80 mm in diameter (Area ~50.28 cm^2) and dried for 2 mins, after which they were put in petri plate of 100mm diameter for each treatment. A control without any EO treatment was prepared. Both the test and control treatments received 10 insects per plate. Parafilm were used to ensure an air tight condition in all the petri plates. Each test and control were replicated three times. Observation of insect survival was recorded at 4 hr, 8 hr, 12 hr and 24 hr. The insects were considered dead if having specific body curvature and no visible motility of any body part (Appendages, Antennae etc.). The effect of binary combination was evaluated using the method described in above para.

1.4.5. Statistical analysis

Abbot's (1925) formula was used to calculate mortality of *S. oryzae*. Probit analysis was used for calculation of LC_{50} values using Finney (1952) method. All data were repeated in three technical replicates and compared by one-way analysis of variance (ANOVA) specifically Tukey's test. Significant difference of percent mortality between control and treatments were analysed at $p<0.01$ and $p<0.05$ using SPSS software (16.0 Version).

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Figure S3 Graph representing mean mortality rate (%) of Black pepper oil against *Sitophilus oryzae*. The exposure of the essential oil was done using the following treatment methods: Fumigant toxicity in without food condition(a) and in with food condition (b); Contact Toxicity in without food condition (c) and with food condition (d) at different time intervals. Each treatment was conducted with three replicates. Data were expressed as mean ± SEM. The means are significantly different between control and treatments using Tukey's test (* $p<0.05$ and ** $p<0.01$).

Figure S4 Fumigant mortality rate (%) of different binary mixtures of Cumin seed and Black pepper essential oils against *Sitophilus oryzae* in without food (a) and with food (b) at different time intervals. Each treatment was conducted with three replicates. Data were expressed as mean ± SEM. The means are significantly different between control and treatments using Tukey's test (* $p<0.05$ and ** $p<0.01$).

Figure S5 Contact mortality rate (%) of different binary mixtures of Cumin seed and Black pepper essential oils against *Sitophilus oryzae* in without food (a) and with food (b) at different time intervals. Each treatment was conducted with three replicates. Data were expressed as mean ± SEM. The means are significantly different between control and treatments using Tukey's test (* $p<0.05$ and ** $p<0.01$).

Table S1 The GC-MS profile of Cumin seed oil showing major constituents.

Compound Name	**RT***	**Mean composition (Area %)**
Acetic acid, oxo-, methyl ester	5.324	0.081
Ethanol, 2-nitro-, propionate (ester)	5.594	0.153
4-Carene, (1S,3R,6R)- (-)-	7.604	0.322
α-Pinene	7.780	1.880
Camphene	8.205	0.188
α -Pinene	**9.095**	**17.869**
α-Pinene	9.545	0.615
Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)-	9.900	0.362
Cyclohexene, 1-methyl-4-(1-methylethylidene)	10.286	0.098
o-Cymene	**10.591**	**25.218**
Cyclohexene, 1-methyl-5-(1-methylethenyl)-, (R)-	10.681	1.222
Eucalyptol	10.741	0.083
γ-Terpinene	**11.656**	**24.525**
o-Isopropenyltoluene	12.511	0.464
Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl) -, (1α, 2α, 5α)-	15.138	0.166
o-Toluic acid, cyclopentyl ester	15.358	0.092
Naphth[2,3-b] oxirene, decahydro-	15.703	0.454
Decalin, anti-1-methyl-, cis-	15.933	0.342
Cuminaldehyde	**17.003**	**15.916**
2-Cyclohexen-1-one, 2-methyl-5-(1-methylethyl)-, (S)-	17.158	0.241
1-Cyclohexene-1-carboxaldehyde, 4-(1-methylethyl)-	17.914	0.150
2-Caren-10-al	18.194	4.132

α -Thujenal	18.354	0.595
Thymol	19.489	0.069
4-Isopropylphenylacetic acid	19.619	0.115
ç-Muurolene	20.730	0.073
Benzoic acid, 3-(1-methylethyl)-	21.820	0.077
1,6,10-Dodecatriene, 7,11-dimethyl-3-methylene-	22.651	0.085
cis-(-)-2,4a,5,6,9a-Hexahydro-3,5,5,9 tetramethyl(1H) benzo cycloheptene	23.161	1.189
Apiol	26.727	0.518

* RT = Retention Time

Table S2 The GC-MS profile of Black pepper oil showing major constituents.

Compound Name	**RT**	**Mean composition (Area %)**
α -Pinene	**7.599**	**1.458**
α -Pinene	**7.805**	**13.600**
Camphene	8.205	0.363
Sabinene	**9.020**	**10.253**
Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-,(1S)-	9.095	7.806
α-Myrcene	9.545	1.736
Bicyclo [3.1.0] hex-2-ene, 4-methyl-1-(1-methylethyl)-	9.910	1.631
3-Carene	10.115	10.464
o-Cymene	10.536	0.604
D- Limonene	**10.706**	**18.089**
4-Carene, (1S,3S,6R)-(-)-	11.286	0.148
4-Carene, (1S,3R,6R)-(-)-	11.591	0.153
Cyclohexene, 1-methyl-4-(1-methylethylidene)	12.421	0.125
Cyclohexene, 1-methyl-4-(1-methylethylidene)-	12.481	0.414
Cyclohexane, 1-methylene-4-(1-methylethenyl)-	12.837	0.206
Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1α, 2α, 5α)	15.132	0.132
Cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-, (3R-trans)	19.614	1.491
Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-	19.934	0.143
α -ylangene	20.655	2.539
α -copaene	21.015	0.241
Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-	21.060	0.245
Caryophyllene	**21.845**	**22.417**

α-Copaene	22.030	0.132
cis- α -Bisabolene	22.651	0.841
α -copaene	23.331	0.244
cis-(-)-2,4a,5,6,9a-Hexahydro-3,5,5,9-tetramethyl(1H) benzocycloheptene	23.726	0.222
α -Muurolene	23.796	0.262
α -Bisabolene	23.971	0.650
Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	24.356	0.975
Longipinocarveol, trans-	25.812	0.476

* RT = Retention Time

Table S3 (a) Effect of binary combinations of cumin seed and black pepper essential oils as fumigant in without food condition on *Sitophilus oryzae* for an exposure period of 24 hours.

Binary Mixture	Ratio	Mortality (%)				X^2	Inference
		Pure compound		Binary mixture			
		O_a	O_b	E	Om		
Cumin seed oil : Black pepper oil	0:100	100	100	-	-	-	-
	20:80	100	100	100	100	0	Additive
	40:60	100	100	100	100	0	Additive
	60:40	100	100	100	100	0	Additive
	80:20	100	100	100	100	0	Additive
	100:0	100	100	-	-	-	-

O_a and O_b represent observed mortality of first and second compound; E_m and Om represent expected and observed mortality. X^2 value (df =1 and $\alpha = 0.05$) is 3.84. If X^2 value < 3.84 then it shows additive effect or else X^2 value > 3.84 shows either synergistic (Om > E) or antagonistic action (Om < E).

Table S3 (b) Effect of binary combinations of cumin seed and black pepper essential oils as fumigant in with food condition on *Sitophilus oryzae* for an exposure period of 72 hours.

Binary Mixture	Ratio	Mortality (%)				X^2	Inference
		Pure compound		Binary mixture			
		O_a	O_b	E	Om		
Cumin seed oil : Black pepper oil	0:100	80	90	-	-	-	-
	20:80	80	90	98	80	3.31	Additive
	40:60	80	90	98	90	0.65	Additive
	60:40	80	90	98	100	0.04	Additive
	80:20	80	90	98	85	1.72	Additive
	100:0	80	90	-	-	-	-

O_a and O_b represent observed mortality of first and second compound; E_m and Om represent expected and observed mortality. X^2 value (df =1 and $\alpha = 0.05$) is 3.84. If X^2 value < 3.84 then it shows additive effect or else X^2 value > 3.84 shows either synergistic (Om > E) or antagonistic action (Om < E).

Table S4 (a) Effect of binary combinations as contact insecticide in without food condition on *Sitophilus oryzae* for an exposure period of 24 hours.

Binary Mixture	Ratio	Mortality (%)				X^2	Inference
		Pure compound		Binary mixture			
		O_a	O_b	E	Om		
Cumin seed oil : Black pepper oil	0:100	60	100	-	-	-	-
	20:80	60	100	100	80	4	Antagonistic
	40:60	60	100	100	95	0.25	Additive
	60:40	60	100	100	100	0	Additive
	80:20	60	100	100	100	0	Additive
	100:0	60	100	-	-	-	-

O_a and O_b represent observed mortality of first and second compound; E_m and Om represent expected and observed mortality. X^2 value (df =1 and $\alpha = 0.05$) is 3.84. If X^2 value < 3.84 then it shows additive effect or else X^2 value > 3.84 shows either synergistic (Om > E) or antagonistic action (Om < E).

Table S4 (b) Effect of binary combinations as contact insecticide in with food condition on *Sitophilus oryzae* for an exposure period of 72 hours.

Binary Mixture	Ratio	Mortality (%)				X^2	Inference
		Pure compound		Binary mixture			
		O_a	O_b	E	Om		
Cumin seed oil : Black pepper oil	0:100	70	100	-	-	-	
	20:80	70	100	100	85	2.25	Additive
	40:60	70	100	100	100	0	Additive
	60:40	70	100	100	100	0	Additive
	80:20	70	100	100	95	0.25	Additive
	100:0	70	100	-	-	-	

O_a and O_b represent observed mortality of first and second compound; E_m and Om represent expected and observed mortality. X^2 value (df =1 and $\alpha = 0.05$) is 3.84. If X^2 value < 3.84 then it shows additive effect or else X^2 value > 3.84 shows either synergistic (Om > E) or antagonistic action (Om < E).

Table S5 Lethal concentration of essential oils against *Sitophilus oryzae* in without and with food conditions.

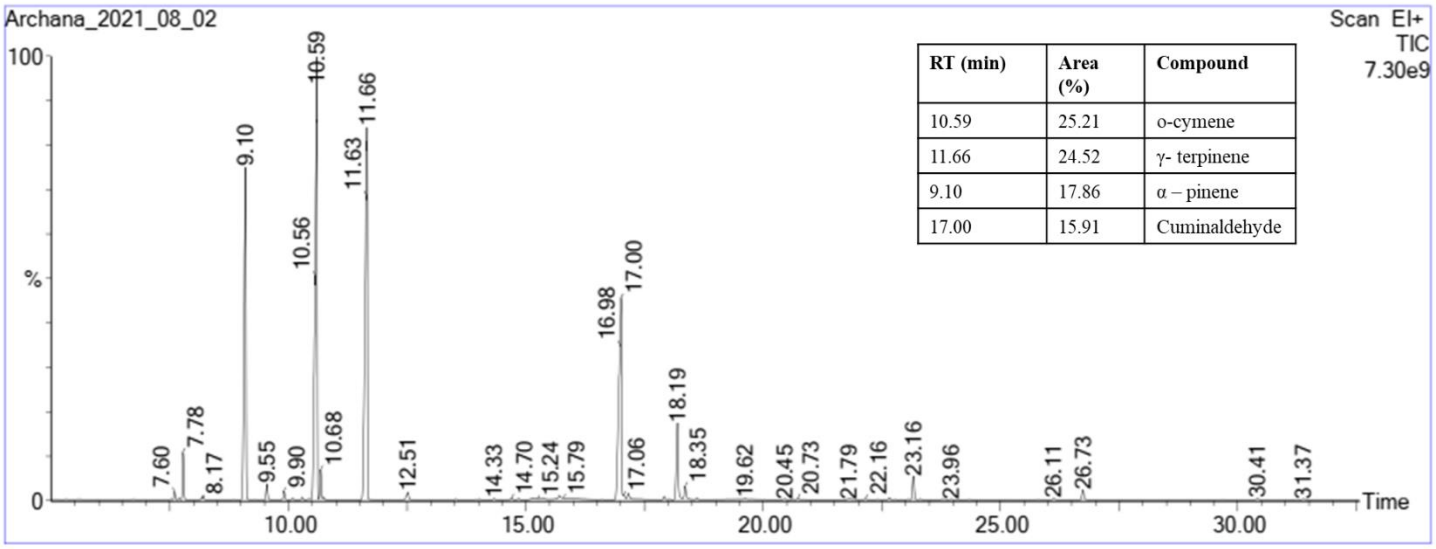
Compound	LC_{50} values with Lower and Upper CL* limits (µL/30ml air)			
	Fumigant Toxicity (µL/30ml air)		Contact Toxicity (µL/ 100cm^3 air)	
	Without Food (24 hr)	With Food (48 hr)	Without Food (24 hr)	With Food (48 hr)
Cumin seed oil	0.6824 (0.238 to 1.95)	23.99 (20.92 to 27.51)	**	15.19 (13.29 to 17.36)
Black pepper oil	2.99 (1.84 to 4.85)	21.31 (16.48 to 27.56)	16.72 (14.26 to 19.61)	18.79 (16.46 to 21.46)

Values indicate the mean (n=3) ± SEM and represent a significant difference between control and treatments at the level of $p<0.05$ and $p<0.01$.

*C.L.: confidence limits which has been calculated with 95% confidence.

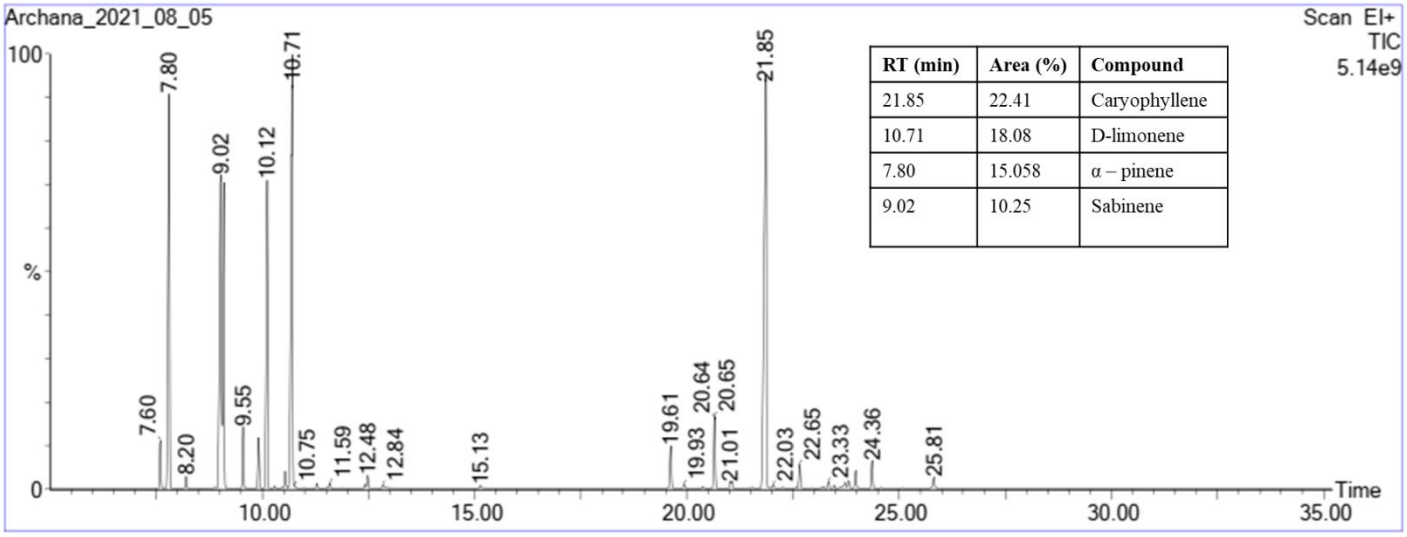
**Represents the record of complete mortality.

(a)



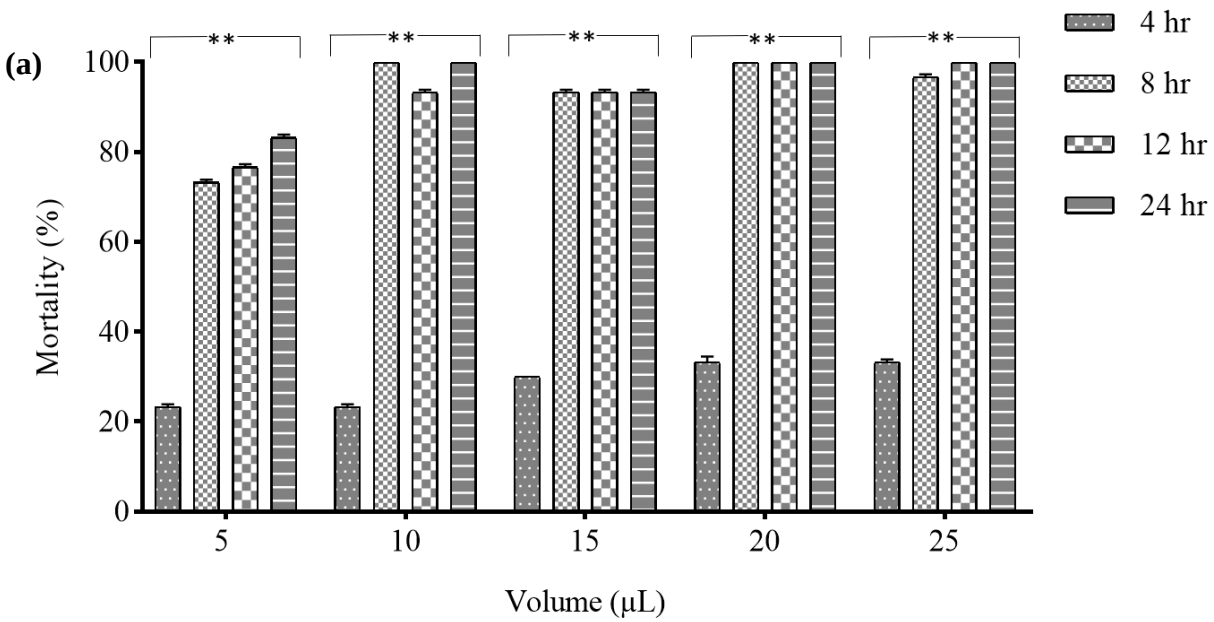
RT (min)	Area (%)	Compound
10.59	25.21	o-cymene
11.66	24.52	γ- terpinene
9.10	17.86	α – pinene
17.00	15.91	Cuminaldehyde

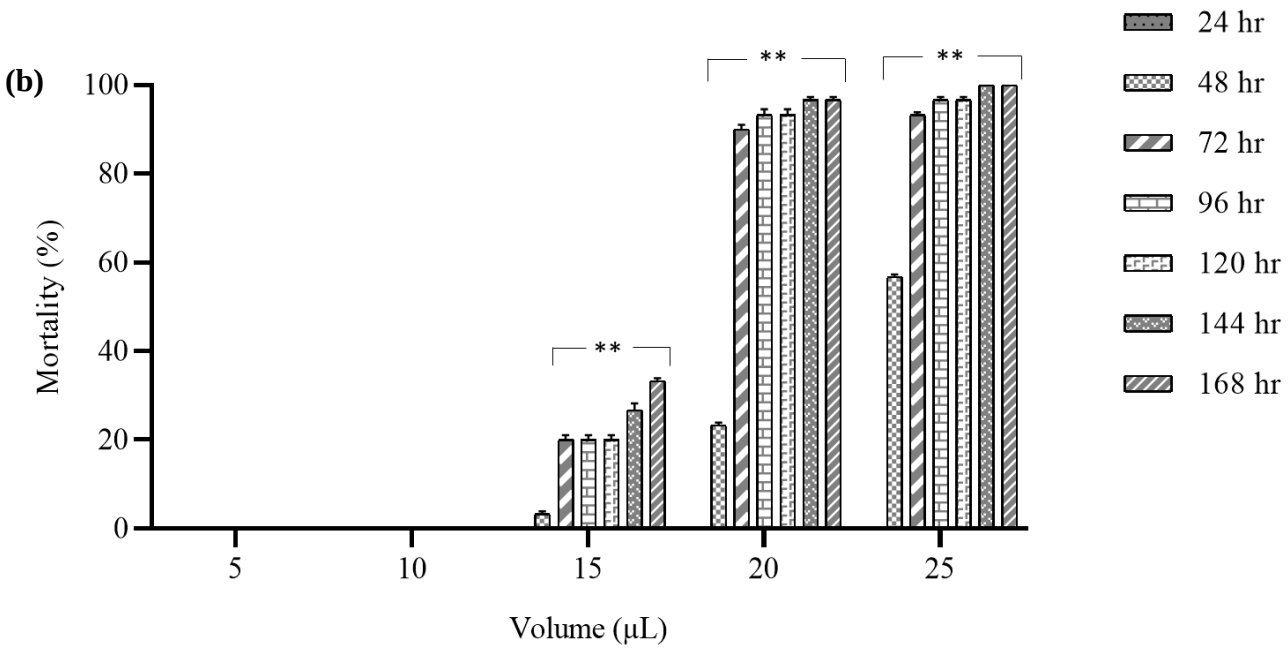
(b)



RT (min)	Area (%)	Compound
21.85	22.41	Caryophyllene
10.71	18.08	D-limonene
7.80	15.058	α – pinene
9.02	10.25	Sabinene

Figure S1 Gas chromatography-Mass spectrometry (GC-MS) analysis of volatilome of essential oils. It represents the chemical profile of essential oils of (a) Cumin seed oil and (b) Black pepper oil.


(a)
Mortality (%)
100
80
60
40
20
0
5
10
15
20
25
Volume (µL)
**
4 hr
8 hr
12 hr
24 hr


(b)
Mortality (%)
100
80
60
40
20
0
5
10
15
20
25
Volume (µL)
**
24 hr
48 hr
72 hr
96 hr
120 hr
144 hr
168 hr

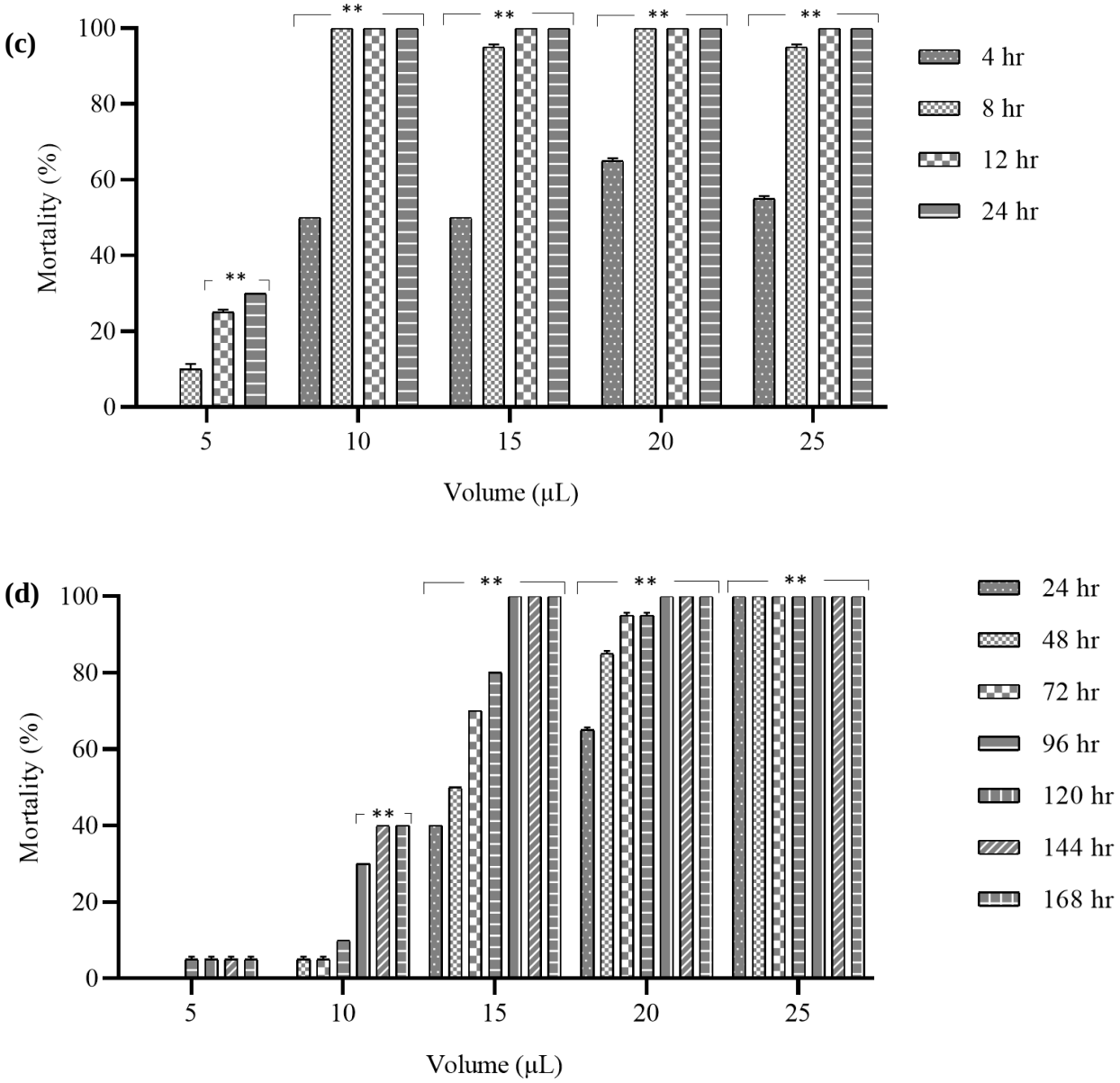
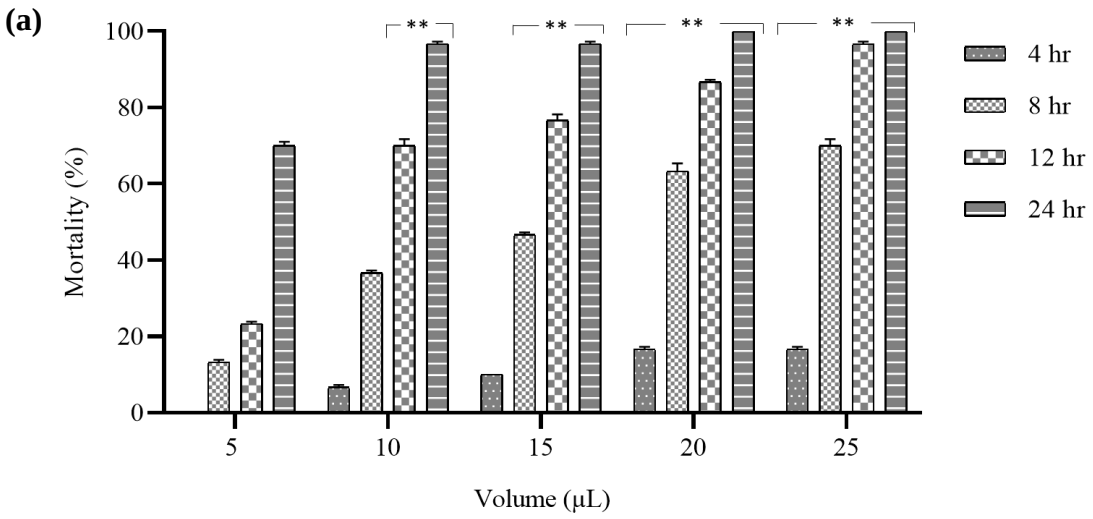
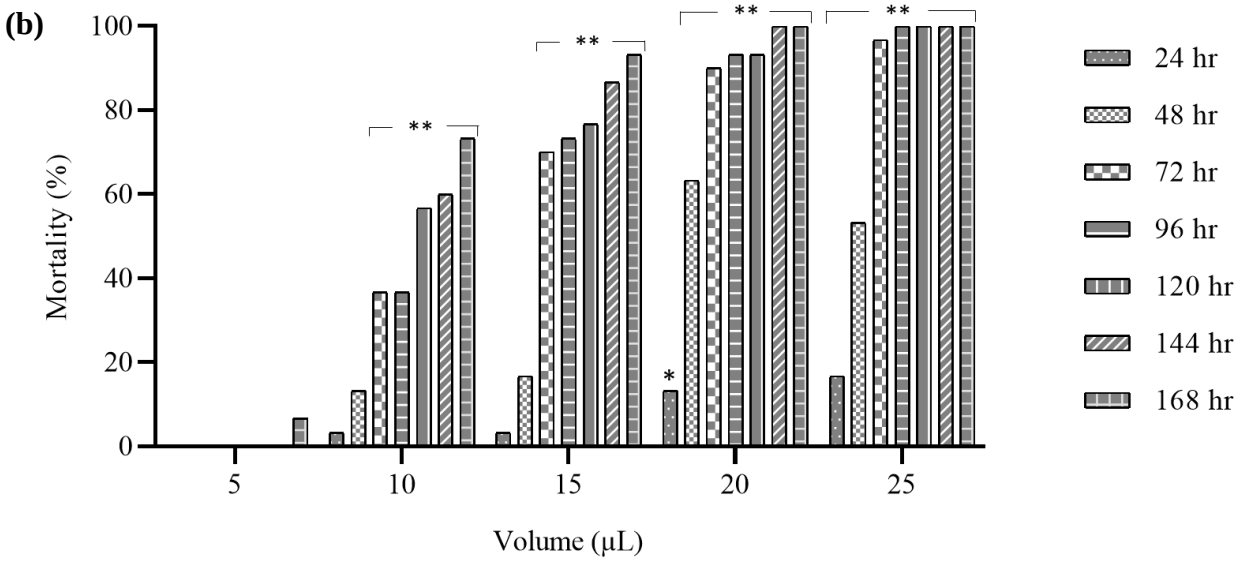


Figure S 2 a Graph representing mean mortality rate (%) of Cumin seed oil against *Sitophilus oryzae*. The exposure of the essential oil was done using the following treatment methods: Fumigant toxicity in without food condition (a) and in with food condition (b); Contact Toxicity in without food condition (c) and with food condition (d) at different time intervals. Each treatment was conducted with three replicates. Data were expressed as mean ± SEM. The means are significantly different between control and treatments using Tukey's test (* $p<0.05$ and ** $p<0.01$).


(a)
Mortality (%)
100
80
60
40
20
0
**
**
**
**
5
10
15
20
25
Volume (µL)
4 hr
8 hr
12 hr
24 hr


(b)
Mortality (%)
100
80
60
40
20
0
**
**
**
**
*
5
10
15
20
25
Volume (µL)
24 hr
48 hr
72 hr
96 hr
120 hr
144 hr
168 hr

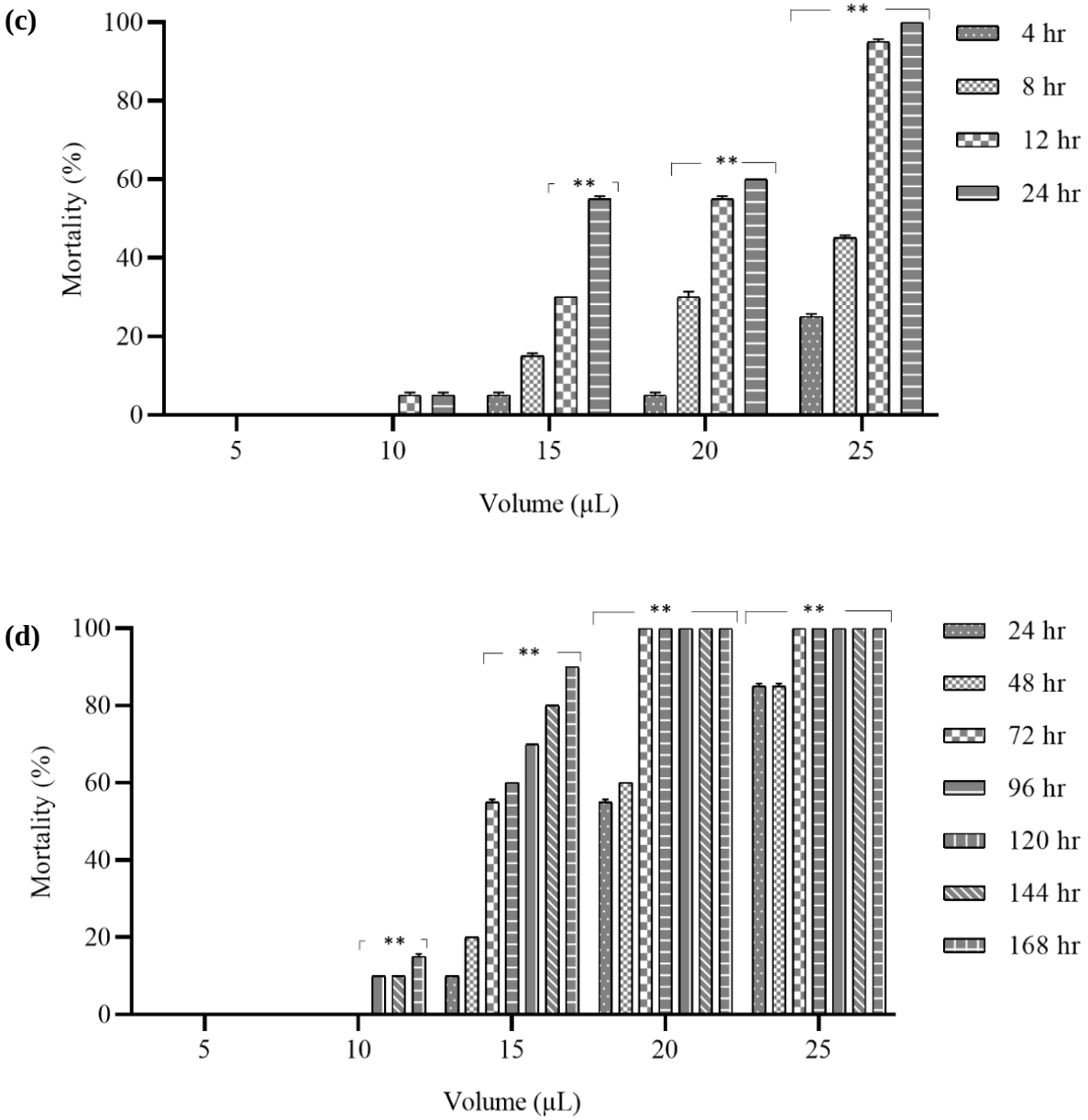


Figure S3 Graph representing mean mortality rate (%) of Black pepper oil against *Sitophilus oryzae*. The exposure of the essential oil was done using the following treatment methods: Fumigant toxicity in without food condition(a) and in with food condition (b); Contact Toxicity in without food condition (c) and with food condition (d) at different time intervals. Each treatment was conducted with three replicates. Data were expressed as mean ± SEM. The means are significantly different between control and treatments using Tukey's test (* $p<0.05$ and ** $p<0.01$).

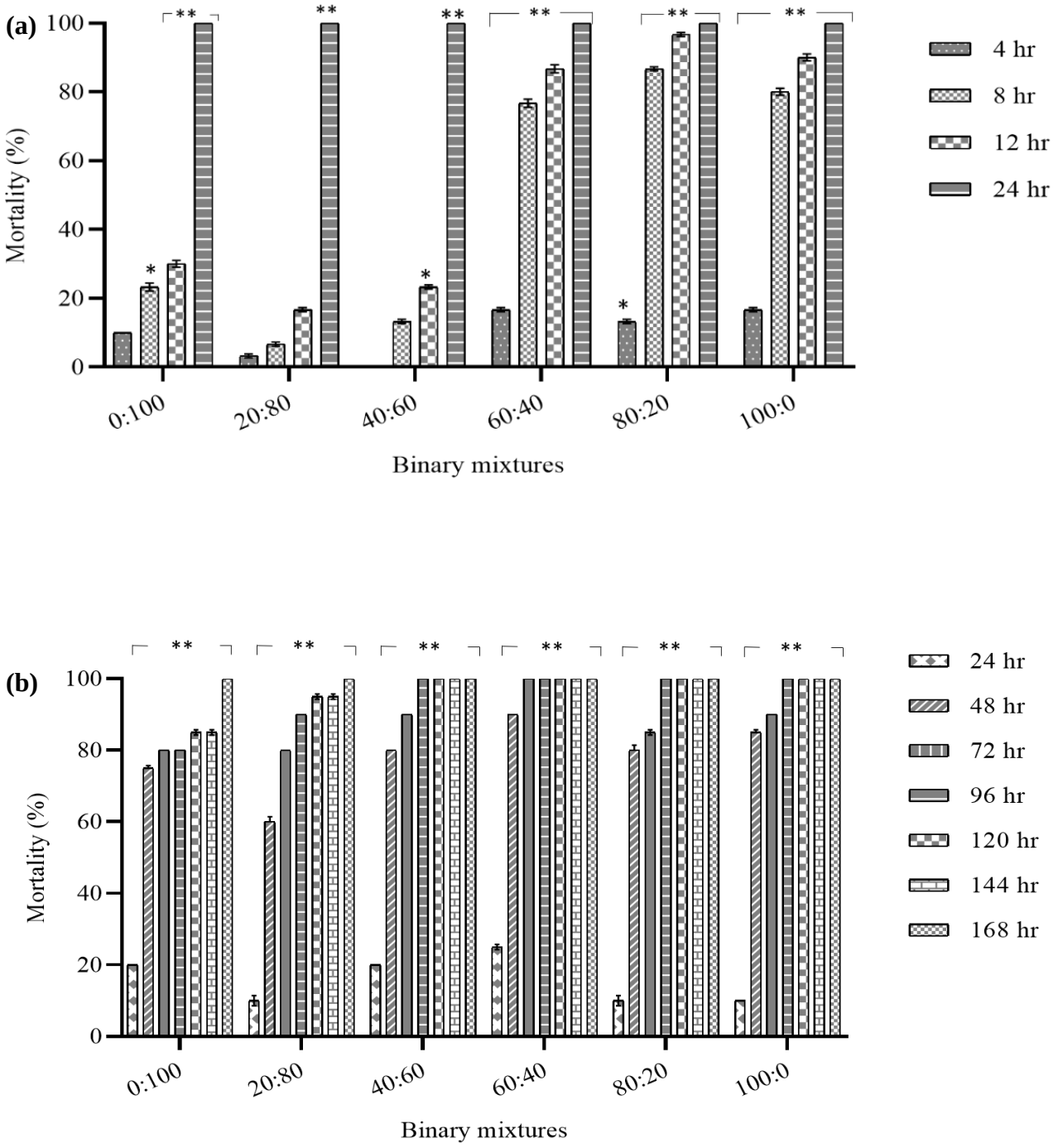


Figure S4 Mean fumigant mortality rate (%) of six binary mixtures of Cumin seed and Black pepper essential oils against *Sitophilus oryzae* in without food (a) and with food (b) at different time intervals. Each treatment was conducted with three replicates. Data were expressed as mean ± SEM. The means are significantly different between control and treatments using Tukey's test (* $p<0.05$ and ** $p<0.01$).

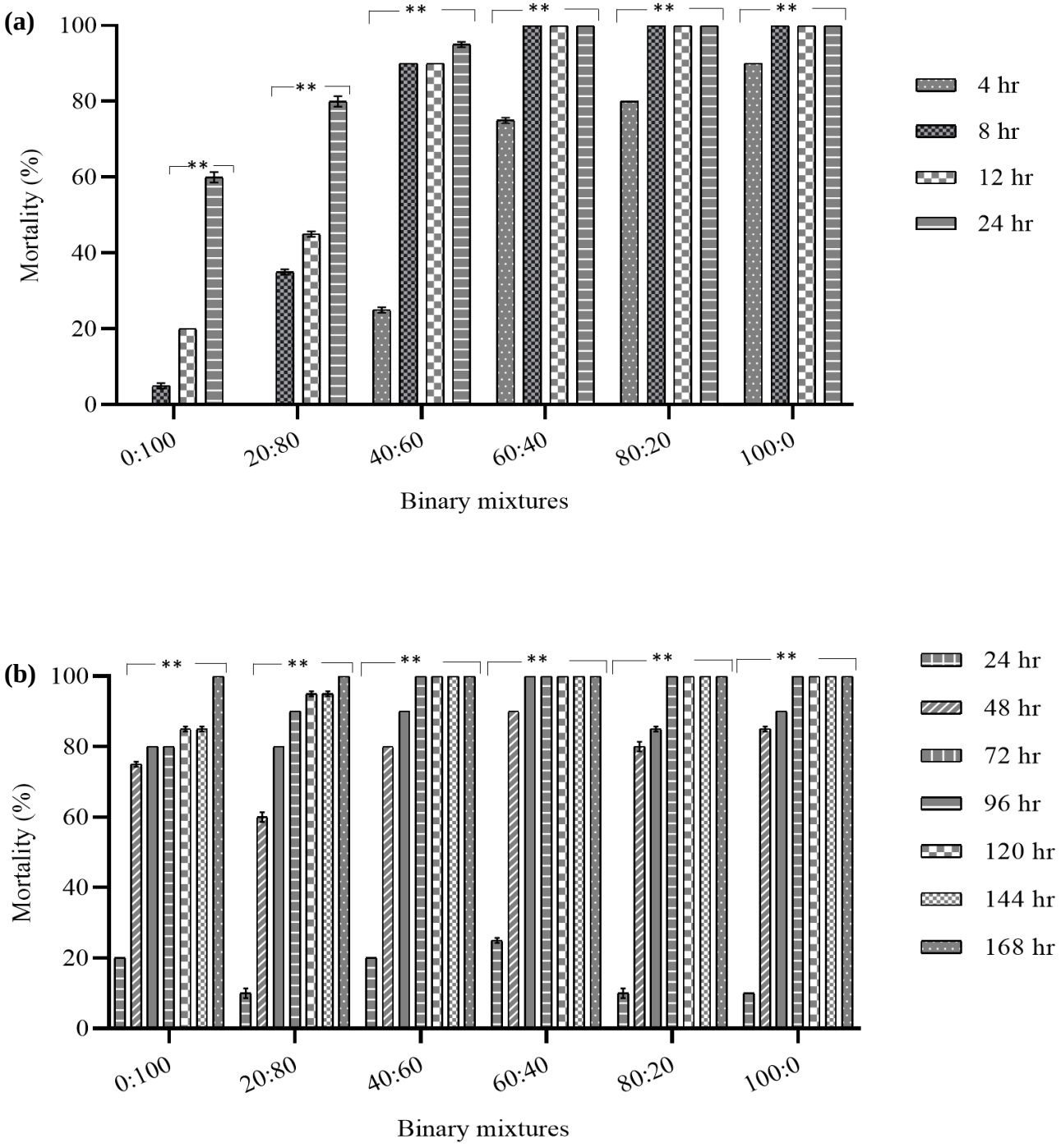


Figure S5 Mean contact mortality rate (%) of six binary mixtures of Cumin seed and Black pepper essential oils against *Sitophilus oryzae* in without food (a) and with food (b) at different time intervals. Each treatment was conducted with three replicates. Data were expressed as mean ± SEM. The means are significantly different between control and treatments using Tukey's test (* $p<0.05$ and ** $p<0.01$).