

Insecticidal efficiency of orange peel (*Citrus sinensis*) essential oil nanoemulsions against *Rhyzopertha dominica* (Fabricius, 1792) and *Tribolium castaneum* (Herbst, 1797)

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Abstract. The insecticidal efficiency of orange (*Citrus sinensis*) peel essential oil (OP-EO) is limited because of its low stability under environmental conditions. Nanoemulsion formulations show promise in overcoming this limitation. Therefore, this study aimed to formulate and characterize the OP-EO nanoemulsion form (OP-EON) and investigate its insecticidal properties against two significant storage pests, *Rhyzopertha dominica* (Fabricius, 1792), and *Tribolium castaneum* (Herbst, 1797). The OP-EON (4:3:3:90 w/w, EO: Tween 80: Ethanol: water) was successfully created using an ultrasonic homogenizer. The major chemical components of the OP-EO were determined to be D-limonene (87.93%), myrcene (3.62%), and α -pinene (1.34%) through GC-MS analysis. The OP-EON was characterized using TEM (50-100 nm), Zeta sizer (the mean droplet particle size of 58.60 nm, the ζ -potential value of -12.6 mV, and the polydispersity index of 0.486), and FT-IR analysis. After 7 days, exposure to 500 ppm of the OP-EON resulted in 50% and 30% mortality rates in *R. dominica* and *T. castaneum*, respectively. Exposure to 1000 ppm of OP-EON resulted in 90% and 55% mortality in *R. dominica* and *T. castaneum*, respectively, after 7 days. Overall, these results clearly showed the potential to exceed the limits of the insecticidal activity of the OP-EO with its nanoemulsion form.

Keywords: essential oil; nanoemulsion; orange peel waste; *Rhyzopertha dominica*; *Tribolium castaneum*

1. Introduction

The citrus industry, which includes fruit juice production, marmalade, jam, jelly, canned food, peel, and essential oil production, holds a significant position in the agricultural industry. However, it also generates substantial amounts of biomass, which poses a significant economic burden (Victor *et al.* 2021). The orange (*Citrus sinensis*) is the most prevalent citrus species in the industry, accounting for approximately 70% of production. However, up to 60% of the processed fruit weight has emerged as waste (Omran *et al.* 2018). Many research has explored the potential uses of orange peel waste products in industries including bioethanol and biogas production, food additives, pharmaceuticals, cosmetics, animal feed, herbal tea, food packaging films, and essential oil production (Shan 2016, Mohanta *et al.* 2021, Panvar *et al.* 2021, Yun and Liu 2022). Essential oil extraction is one of the widely used bio-economical methods for the evaluation of these wastes, considering the rich functional ingredients of the orange peel (Gavahian *et al.* 2019, Siddiqui *et al.* 2022).

Orange peel essential oil (OP-EO) has antifungal, antioxidant, anti-aflatoxicogenic, and antimicrobial properties, making it a popular ingredient in various industries. It is commonly used in foods including beverages, ice cream,

cakes, and film coatings, pharmacology products such as air fresheners, household products, perfumes, and detergent industry (Geraci *et al.* 2017, Alparslan *et al.* 2019, Gültepe 2020, Sheikh *et al.* 2021, Khamsaw *et al.* 2022). There have been many studies conducted on the insecticidal efficiency of OP-EO against various pests, including *Tribolium confusum*, *Callosobruchus maculatus*, *Sitophilus oryzae*, *Sitophilus granarius*, *Oryzaephilus sp.*, *Rhyzopertha dominica*, *Zabrotes subfasciatus*, *Cimex camicidae*, *Bemisia tabaci* biotype B, *Musca domestica*, and *Sitophilus zeamais* (de Carvalho Ribeiro *et al.* 2010, Zewde and Jembere 2010, Kumar *et al.* 2012, Tandorost and Karimpour 2012, Oboh *et al.* 2017, Mursiti *et al.* 2019, Oyedeji *et al.* 2020, Achimón *et al.* 2022). Although the insecticidal effectiveness of OP-EOs has been proven by the above-mentioned studies and promises a serious bio-economic income thanks to their functional ingredients, their use and commercialization in the field as an insecticide and is controversial because of the volatility of essential oils and their low stability against abiotic and biotic stress factors. Therefore, alternative approaches are gaining attention to harness the high insecticidal potential of essential oils (Menossi *et al.* 2021, Esmaili *et al.* 2021). Nanoemulsion structures are gaining attention due to their many advantages, including kinetic stability, temperature and pH stability, increased efficiency, and controlled release of essential oils (Cicek and Nadaroglu 2015, Ameer *et al.* 2022, Ozogul *et al.* 2022). Although there have been studies on the insecticidal efficiency of orange peel essential oil nanoemulsion (OP-EON) against *Tribolium confusum*, *Cryptolestes ferrugineus*,

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Table 1 GC-MS process parameters for the OP-EO

Analytical Line 1		Analytical Line 2	
Column Oven Temp.	50.0 °C	Column Oven Temp.	100.00 °C
Injection Temp.	280.00 °C	Injection Temp.	Split
Injection Mode	Split	Injection Mode	Pressure
Flow Control Mode	Linear Velocity	Flow Control Mode	83.7 kPa
Pressure	53.6 kPa	Pressure	4.0 mL/min
Total Flow	29.0 mL/min	Total Flow	1.00 mL/min
Column Flow	1.00 mL/min	Column Flow	25.4 cm/sec
Linear Velocity	36.3 cm/sec	Linear Velocity	3.0 mL/min
Purge Flow	3.0 mL/min	Purge Flow	0.0
Split Ratio	25.0	-	-
GC Program		MS Table	
Ion Source Temp.	240.00 °C	Start Time	3.00 min
Interface Temp.	250.00 °C	End Time	57.00 min
Solvent Cut Time	3.00 min	ACQ Mode	Scan
Detector Gain Mode	Relative to the Tuning Result	Event Time	0.30 sec
Detector Gain	1.06 kV +0.00 kV	Scan Speed	2000
Threshold	0	Start m/z	40.00
-	-	End m/z	600.00

Culex pipiens, and *Aphis gossypii*, no research has been found on its insecticidal activity against *Rhyzopertha dominica* (Fabricius, 1792) and *Tribolium castaneum* (Herbst, 1797). These two pests are major global threats to cereal grains (Azmy *et al.* 2019, Giunti *et al.* 2019, Laudani *et al.* 2022, Sogan *et al.* 2023).

Rhyzopertha dominica (Fabricius 1792) (Coleoptera: Bostrichidae) and *Tribolium castaneum* (Herbst 1797) (Coleoptera: Tenebrionidae) are commonly referred to as the lesser grain borer and the red flour beetle, respectively. These pests cause significant economic damage to stored grain. They exhibit resistance to chemical insecticides such as phosphine, bifenthrin, pirimiphos-methyl, and deltamethrin (Wang *et al.* 2020, Attia *et al.* 2020, Ortega *et al.* 2021, Oppert *et al.* 2022). Taking into account the comments above, this study aims to establish and describe the OP-EON and examine its insecticidal effectiveness against *R. dominica* and *T. castaneum*.

2. Materials and methods

2.1 Materials

The OP-EO was purchased from a local company, BIOMESI Bioagrotechnology R&D, located in Adana, Turkey (Durmuş *et al.* 2023). *R. dominica* and *T. castaneum* were obtained from the Laboratory of Toxicology, Department of Plant Protection at Atatürk University in Erzurum, Turkey. They were then transferred to 5 L glass jars containing both intact and broken durum wheat (*Triticum durum* Desf.). The mixture of flour and bran (in a ratio of 1:1) was cultured at 28±1 °C and 50 ±5% relative humidity of (for *R. dominica*), 28±2 °C and 70 ±5% (for *T.*

castaneum) relative humidity with 12:12 hours (light: dark) (Giunti *et al.* 2021, Lima *et al.* 2021). The products used to feed the insects were stored at -20°C and purified to remove any existing pests. These pests less than 2 weeks- old were used in the experiments.

2.2 Gas chromatography–mass spectroscopy (GC–MS) analysis of the OP-EO

The chemical composition of the OP-EO was determined through service procurement at the Atatürk University Eastern Anatolia High Technology Application and Research Center (DAYTAM) in Erzurum, Turkey. The OP-EO was analyzed using a Shimadzu QP2010 ULTRA gas chromatography-mass spectrometry (GC-MS) equipment (Shimadzu Corporation, Kyoto, Japan). The GC was equipped with a 30-meter long 5-MS type column and utilized 70 eV electron impact ionization (Dağalan *et al.* 2021). The GC conditions involve maintaining a temperature of 50°C for 1 min, which is then increased to 300°C over a period of 15 min. The temperatures of the split injector and ion source were 280°C and 240°C, respectively (Çiçek *et al.* 2024). The FFNSC 3 and W9N11 libraries were used to characterize the compounds. The process parameters for GC-MS from DAYTAM are presented in Table 1.

2.3 Formulation of the OP-EON

The nanoemulsion formulation of OP-EO was prepared following the method described by Durmuş (2020) with slight modifications. The OP-EON was created using an ultrasonic homogenizer (Bandelin Sonopuls, BANDELIN electronic GmbH & Co. KG, Berlin, Germany) with a

Table 2 Treatment groups with related compositions

Treatment Groups	Dose	
	500 ppm	1000 ppm
Control	Water (80%) + Ethanol (20%)	Water (50%) + Ethanol (50%)
Tween 80	Tween 80 (0.6%) + Water (79.52%) + Ethanol (19.88%)	Tween 80 (0.6%) + Water (49.7%) + Ethanol (49.7%)
OP-EO	Essential Oil (25%) + Water (50%) + Ethanol (25%)	Essential Oil (33,3%) + Water (33,3%) + Ethanol (33,3%)
OP-EON	Nanoemulsion (25%) + Water (50%) + Ethanol (25%)	Nanoemulsion (33,3%) + Water (33,3%) + Ethanol (33,3%)
Positive control (Deltamethrin)	5 ppm	5 ppm

titanium probe that has a diameter of 2 mm and a height of 1950 mm (MS72). 1.2 mL of OP-EO, 0.9 mL of Tween 80, 0.9 mL of ethanol, and 27 mL of water were added to a 50 mL glass beaker, resulting in a total volume of 30 mL. The mixture was then exposed to ultrasonication at 70 amplitude, 20 kHz, and 500 W for 15 minutes (Çiçek *et al.* 2024). The beaker was placed in a container filled with ice to maintain a constant temperature and safeguard the nanoemulsion from any negative impacts caused by the heat generated during the process (Durmuş 2020).

2.4 Morphological and structural characterization of the OP-EON

The surface morphology, drop size, drop size distribution, zeta potential (ζ -potential), polydispersity index (PDI), and molecular characterization of the OP-EON were determined using various techniques. The transmission electron microscope (TEM) (Hitachi HighTech HT7700, Japan) and zeta sizer (Malvern Zetasizer Nano ZSP, Malvern Instruments Pvt Ltd, UK) were used to determine drop size, drop size distribution, and ζ -potential. Fourier Transform Infrared Spectroscopy (FTIR) (Bruker VERTEX 70v brand, Germany) was used to analyze the molecular characteristics of the OP-EON. These techniques were employed in the DAYTAM (Adak *et al.* 2020, Norcino *et al.* 2020). Before capturing the TEM image of OP-EON, it was treated with uranyl acetate, a negative electron stain, for 20 seconds. After that, it was washed three times with deionized water and left to dry on a carbon-coated grid overnight at room temperature (Chuesiang *et al.* 2018).

2.5 Insecticidal efficiency of the OP-EON

The insecticidal efficiency of OP-EON against *R. dominica* and *T. castaneum* was investigated using two different doses: 500 ppm and 1000 ppm. The process was carried out in Petri dishes, in which pre-prepared blotting papers were placed. 90 μ L of each treatment group listed in Table 2 were thoroughly mixed with 30 grams of wheat in 2.0 mL Eppendorf tubes for 10 minutes. Wheat samples were mixed with treatment groups and 20 pests and then placed in pre-labeled Petri dishes. I wrapped the edges of the Petri dishes with Parafilm. The experiment was repeated three times. Pest deaths were monitored and recorded for seven days, starting with measurements taken, on the 1st, 2nd, 4th, 8th, 12th, 16th, and 24th hours on the first day,

and then every 24 hours on the subsequent days (Kavallieratos *et al.* 2022). The treatment groups in this study (Table 2) were prepared, based on the research conducted by Kavallieratos *et al.* (2022).

2.6 Data analysis

All data related to insecticide activity were analyzed using IBM Corp.'s SPSS 20.0 statistical software (Armonk, NY, USA). Post-hoc and Duncan's analyses were conducted to examine the means of homogeneous data and determine significance at the $p \leq 0.05$ level. Furthermore, a multivariate test was conducted to assess the impact of between-subject factors (insect, treatment, concentration, time) on mortality percentage (%) (Kavallieratos *et al.* 2022).

3. Results and discussions

3.1 Gas chromatography–mass spectroscopy (GC–MS) analysis of the OP-EO

The composition of the compound identified by GC-MS for the OP-EO is presented in Table 3, which lists thirty compounds identified in the OP-EO. The chemical composition of the OP-EO is dominated by D-limonene (87.93%), myrcene (3.62%), and α -pinene (1.34%). The essential oil contained several constituents, including linalool (0.87%), sabinene (0.86%), decanal (0.56%), 2,6-bis(1,1-dimethylethyl)-4-methylphenol (0.54%), γ -terpinene (0.50%), and 1-methyl-4-(1-methylethenyl)-1,2-cyclohexanediol (0.48%). While the major components of essential oil contribute to its insecticidal activity, the minor components can have a synergistic effect on this activity (Giunti *et al.* 2019). In another study, the main components of OP-EO were found to be limonene (68.86%), carveol (4.69%), carvone (3.47%), limonene-1,2-diol (2.45%), and α -pinene (1.45%). The minor constituents of OP-EO included isopiperitenol (0.63%), bisabolene epoxide (0.17%), isocarveol (0.20%), sinensal beta (1.05%), (+)-3-carene (0.25%), linalool (0.45%), and myrcene (0.08%) (Sogan *et al.* 2023). The chemical composition of OP-EO varies depending on its geographical distribution, genetic factors, age, extraction procedures, environmental and agronomic practices, ripening stage, and time of harvest. However, the studies on the OP-EO reported that D-limonene (65.3–97.0%) was the main major constituent (Geraci *et al.* 2017,

Table 3 Chemical composition of the OP-EO

Peak	RT ^a	RI ^b	Compound	Formula	Areas %	Height %
1	7.100	933, 0, 948	α -pinene	C ₁₀ H ₁₆	1.34	3.90
2	8.025	972, 0	Sabinene	C ₁₀ H ₁₆	0.86	2.44
3	8.127	978, 943, 0	β -Pinene	C ₁₀ H ₁₆	0.17	0.45
4	8.397	991, 0, 958	Myrcene	C ₁₀ H ₁₆	3.62	9.98
5	8.660	0, 1005	Octanal	C ₈ H ₁₆ O	0.31	0.87
6	8.923	0, 1009, 948	DELTA.3-Carene	C ₁₀ H ₁₆	0.28	0.68
7	9.270	1025, 1042	Cymene <para->	C ₁₀ H ₁₄	0.27	0.44
8	9.507	1018, 0, 1030	D-Limonene	C ₁₀ H ₁₆	87.93	67.54
9	9.802	1046, 976, 0	β -Ocimene	C ₁₀ H ₁₆	0.08	0.23
10	10.108	1058, 998, 0	γ -Terpinene	C ₁₀ H ₁₆	0.50	1.42
11	11.060	1101, 1104, 1082, 0	Linalool	C ₁₀ H ₁₈ O	0.87	2.34
12	11.161	0, 1104	Nonanal	C ₉ H ₁₈ O	0.08	0.22
13	11.622	1122, 1120, 1140,	Mentha-2,8-dien-1-ol <trans-, para->	C ₁₀ H ₁₆ O	0.29	0.79
14	11.970	1138, 0, 1140	Mentha-2,8-dien-1-ol <cis-, para->	C ₁₀ H ₁₆ O	0.19	0.45
15	12.063	1031, 0	7-Oxabicyclo[4.1.0]heptane, 1-methyl-4-(1-methylethenyl)-	C ₁₀ H ₁₆ O	0.16	0.43
16	12.373	1125, 1152, 0	6-Octenal, 3,7-dimethyl-, (R)-	C ₁₀ H ₁₈ O	0.10	0.28
17	13.269	1189, 1201, 0	Isocarveol <trans->	C ₁₀ H ₁₆ O	0.11	0.34
18	13.355	1143, 0	α -Terpineol	C ₁₀ H ₁₈ O	0.16	0.45
19	13.503	1261, 0, 1140	(S)-(-)-(4-Isopropenyl-1-cyclohexenyl)methanol	C ₁₀ H ₁₆ O	0.09	0.21
20	13.626	1208, 0, 1204	Decanal <n->	C ₁₀ H ₂₀ O	0.56	1.39
21	14.009	1206, 1223, 0	2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-, cis-	C ₁₀ H ₁₆ O	0.10	0.25
22	14.219	1201, 1230, 0, 1189	cis-p-mentha-1(7),8-dien-2-ol	C ₁₀ H ₁₆ O	0.11	0.30
23	14.625	1246, 1190, 0	Carvone	C ₁₀ H ₁₄ O	0.10	0.26
24	16.773	1346, 0, 1325, 1386	1,2-Cyclohexanediol, 1-methyl-4-(1-methylethenyl)-	C ₁₀ H ₁₈ O ₂	0.48	1.20
25	18.024	1432, 0, 1398, 1490	Elemene <gamma->	C ₁₅ H ₂₄	0.10	0.20
26	18.239	1410, 0, 1402, 1516	Dodecanal <n->	C ₁₂ H ₂₄ O	0.15	0.34
27	20.239	1474, 0	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1R-(1.alpha.,7.beta.,8a.alpha	C ₁₅ H ₂₄	0.24	0.58
28	20.514	0, 1668, 1503	Phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl-	C ₁₅ H ₂₄ O	0.54	1.44
29	24.084	0, 1701, 1646, 1579	β - Sinensal	C ₁₅ H ₂₂ O	0.10	0.27
30	33.478	1666, 0, 1377	9-t-Butyl-4-iodo-2,2-dimethyladamantane	C ₁₆ H ₂₇ I	0.13	0.32

^aRetention Time, ^bRetention Index

Giunti *et al.* 2019, Bento *et al.* 2020, Kumar *et al.* 2020, Van Dat *et al.* 2020).

3.2 Morphological and structural characterization of the OP-EON

Detailed characterization is necessary to confirm the presence of nanostructures in the production of nano-materials. Therefore, in this study, TEM, Zetasizer, and FTIR were utilized for the characterization of the OP-EON.

During the nanoemulsion process, the reaction mixture turned milky white (as observed macroscopically) after 7 min of ultrasonication. Eventually, this mixture became translucent and dispersed. TEM images of the OP-EON are shown in Fig. 1. The images indicate that the OP-EON are spherical droplets with a size range of 50-100 nm. Negative staining, such as uranyl acetate, is utilized to visualize the outer layer of nanoemulsion droplets in situations where cryo-FESEM or TEM facilities are not accessible (Klang *et al.* 2012). According to Fig. 1, the OP-EON appears to be

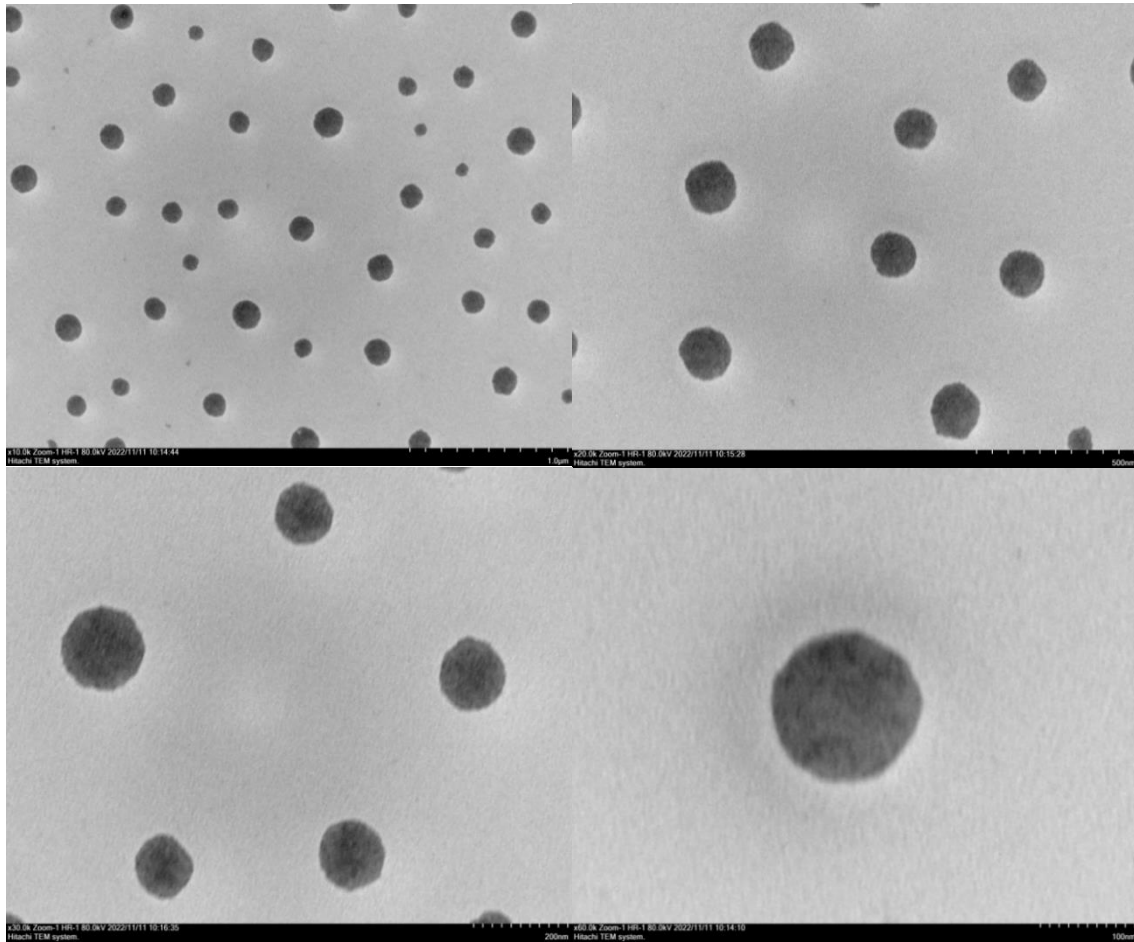


Fig. 1 TEM images of the OP-EON at different magnifications (10.0 kx, 20.0 kx, 30.0 kx, and 60.0 kx, respectively)

spherical and smooth, without any physical deformations or agglomerations. Furthermore, as the TEM images were captured while the droplets were in a desiccated state, it is probable that smaller droplet sizes were detected in comparison to the results obtained from DLS analysis. Similar results were reported by Rochín-Wong *et al.* (2018).

Zetasizer analysis was performed to measure the particle size, zeta potential values, and polydispersity indexes of the OP-EON droplets at a constant temperature of 25°C (as shown in Fig. 2). According to Fig. 2, the mean particle size of droplets in the OP-EON was 58.60 nm ($n=3$). This size is higher than the sizes previously reported by Das *et al.* (2020) and Sugumar *et al.* (2016), which were 43.23 nm and 16 nm, respectively. This finding is consistent with the presence of nanoemulsions ranging from 50 to 100 nm in size, as observed in the TEM image. However, it does not support the possibility of nanoemulsions smaller than 50 nm. When considering the size distribution by intensity, droplet particle sizes started at less than 10 nm. Nanoemulsions with sizes ranging from 20 nm to 120 nm occupied a significant percentage of the intensity field, which strongly supported the size distribution observed in TEM images.

The polydispersity index (PDI) shows the uniformity and stability of the size of the nano droplets. According to Hashem *et al.* (2018), PDI values of ≤ 0.25 demonstrate a narrow droplet size distribution, which suggests excellent

long-term stability of the nanoemulsion. Furthermore, according to Mohammed *et al.* (2020), PDI values greater than 0.5 indicate a wide range of particle sizes. In this study, the mean PDI value of the OP-EON was recorded as 0.486. The dynamic nature of the systems was demonstrated by this value, indicating the presence of bicontinuous structures (Mostafa *et al.* 2015).

The zeta potential (ZP) was measured to determine the strength of the surface electrostatic interaction of the OP-EON. The average ZP value of The OP-EON was measured to be -12.6 mV. Similarly, Farouk *et al.* (2022) reported that the zeta potential value of OP-EON (97.22 \pm 0.13 nm) prepared using Polysorbate 20 was -16.31 \pm 2.54 mV. The researchers associated this value with the presence of anionic groups in fatty acids and glycols found in the surfactant used in the study. In a separate study, Das *et al.* (2020) reported that the particle charge of the nanoemulsion of orange peel essential oil (CSALG-NE), which was prepared using sodium alginate and Tween 80, was recorded as -16.32 mV. They stated that this negatively charged surface provides sufficient electrostatic repulsion, which is crucial for maintaining the stability of the nanoemulsion. Somala *et al.* (2022) stated that a zeta potential greater than +30 mV or less than -30 mV confirms the stability of the nanoemulsion, indicating a high energy barrier against the coalescence of dispersed droplets. The non-ionic structure of Tween 80, combined with the

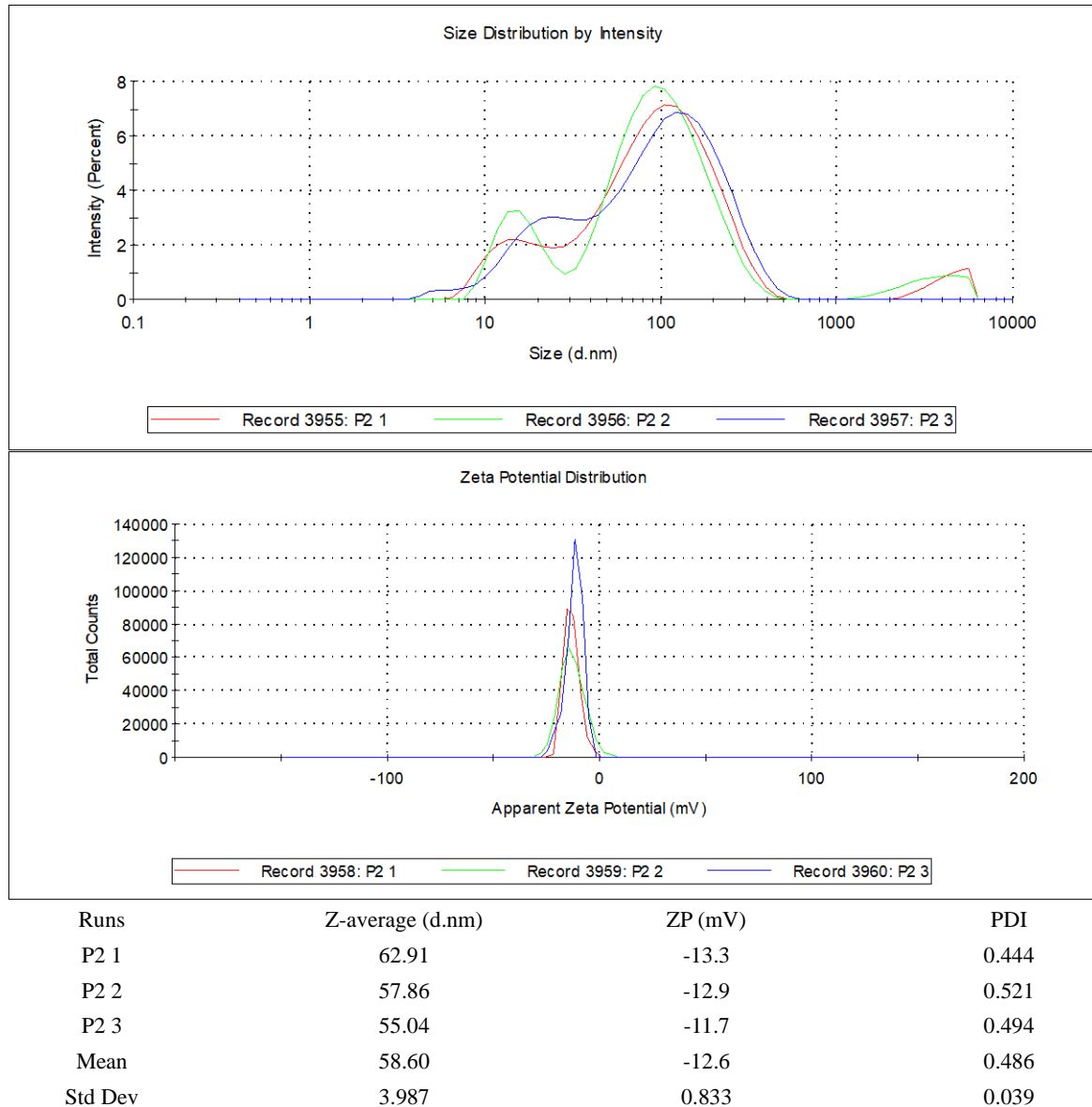


Fig. 2 The droplet particle size distribution, zeta potential distribution, polydispersity indexes of the OP-EON (P2 1, P2 2, P2 3: three repetitions, ZP: Zeta potential, PDI: Polydispersity index)

mechanical stress applied during the ultrasonication process used in this study, may have caused an increase in the release of negatively charged -OH- and -COOH- groups from the essential oil onto the surface of the nanoemulsion, resulting in a negative ZP value.

FTIR analysis was performed to characterize the molecular structure and functional groups of the OP-EON, as shown in Figure 3. The FTIR spectra of the OP-EON showed a strong signal at 1246 cm^{-1} corresponding to the C-O-C stretch, and a peak around 1101 cm^{-1} attributed to the C-OH and C-O-C stretches. The FTIR spectrum exhibits peaks at 538 cm^{-1} , 572 cm^{-1} , and 609 cm^{-1} , which correspond to stretches in C-Br and C-I halo compounds. Additionally, it is evident that the two peaks at 725 cm^{-1} and 850 cm^{-1} indicate C-H and C=C stretch, respectively, which suggests the presence of a monosubstituted benzene derivative, 1,3-disubstituted benzene, 1,2,3-trisubstituted alkene, 1,2,4-trisubstituted benzene, and 1,3-disubstituted

alkene. A series of peaks were found at 946 cm^{-1} (C=C, monosubstituted alkene), 1245 cm^{-1} (C-N, amine and C-O, alkyl aryl ether), and 1299 cm^{-1} (C-O, aromatic ester and C-N, aromatic amine). The absorption spectrum displays several peaks, indicating different functional groups. The peak at 1458 cm^{-1} corresponds to C-H stretching in alkane and methylene groups. The peak at 1953 cm^{-1} indicates C-H stretching in aromatic compounds and C=C=C allene. Finally, the peak at 2736 cm^{-1} represents a C-H stretching aldehyde doublet. The wavelengths of the peaks observed in this study are similar to the FTIR patterns found in other studies on orange peel essential oil and its nanoemulsion form (Das *et al.* 2020, Kumar *et al.* 2020). In this study, the peak at 1733 cm^{-1} indicates the C=O stretching of aldehyde, which may be associated with the presence of ester groups in Tween 80. Similarly, the same results were reported by Osanloo *et al.* (2022). In addition, the bands at 2856 cm^{-1} and 2921 cm^{-1} represent the C-H stretching (alkane) while

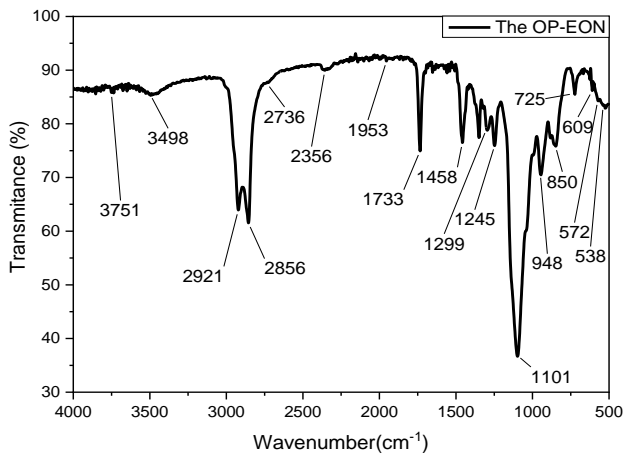


Fig. 3 FTIR spectra of the OP-EON

Table 4 Multivariate analysis of variance parameters for the main effects and their associated interactions that resulted in the recorded mortalities of *R. dominica* and *T. castaneum* between and within exposures (Error df = 31)

Between exposure intervals	df	F	P
Insect	1	63.967	.000
Treatment	3	285.244	.000
Concentration	1	151.154	.000
Insect x Treatment	3	18.759	.000
Insect x Concentration	1	7.143	.012
Treatment x Concentration	3	44.378	.000
Insect x Treatment x Concentration	3	5.273	.005
Within exposure intervals	df	F	P
Time	12	1682.05	.000
Time x Insect	12	63.671	.000
Time x Treatment	36	377.295	.000
Time x Concentration	12	179.248	.000
Time x Insect x Treatment	36	20.038	.000
Time x Insect x Concentration	12	29.053	.000
Time x Treatment x Concentration	36	62.641	.000
Time x Insect x Treatment x Concentration	36	21.262	.000

the band at 3498 cm^{-1} indicates the N-H stretching (primary amine). These peaks may be attributed to the use of Tween 80 in the nanoemulsion formulation (Hasanzadeh *et al.* 2022). The peak at 3751 cm^{-1} demonstrates the stretching of the hydrogen bond -OH.

3.3 Insecticidal efficiency of the OP-EON

Based on Table 4, all components of the study (insects, treatments, concentrations, time, and mortality) and their corresponding interactions exhibited significant differences between and within exposure intervals.

After 8 hours, exposure to 1000 ppm of OP-EON resulted in 5% mortality for *R. dominica*. After 24 hours, the same dose resulted in a 60% mortality rate for this insect. However, when treated with 500 ppm of OP-EON and 500

ppm or 1000 ppm of OP-EON, the mortality rates were lower (10%, 10%, and 20%, respectively) at this treatment interval. In *R. dominica*, exposure to 500 ppm of OP-EON resulted in 75% mortality, whereas exposure to 500 ppm of OP-EON caused 50% mortality after 168 hours. The highest mortality rate (98.33%) was achieved with the application of 1000 ppm of OP-EON treatment after 7 days (Table 5). Mahdi and Behnam (2018) reported that the essential oil extracted from orange leaves (*Citrus sinensis*) exhibited insecticidal activity against *R. dominica*, with an LC_{50} value of $90.15\text{ }\mu\text{L/L}$ air, after 24 hours of treatment. In this study, treatment with 1000 ppm of OP-EON resulted in 90% mortality. Furthermore, the results indicate that mortality rates increased as the concentrations and duration of exposure to the OP-EON and OP-EON treatment groups increased. Mohammed and Nasir (2020) reported that after 24, 48, and 72 hours, $10\text{ }\mu\text{L/mL}$ of eucalyptus oil nanoemulsion resulted in mortality rates of $53.3\pm 0.88\%$, $73.3\pm 0.88\%$, and $83.3\pm 0.88\%$, respectively, in *R. dominica*. Igbal *et al.* (2022) reported that nanosuspensions of *Azadirachta indica* (with a size of $275.8\pm 0.95\text{ nm}$) caused a mortality rate of $84.97\pm 2.83\%$ in *R. dominica*, compared to the crude extract. These findings suggest that the insecticidal activity of essential oil nanoemulsions is influenced by various factors, including differences in chemical composition, size, concentration, and treatment duration.

In *T. castaneum*, exposure to 500 ppm of OP-EON resulted in 10% mortality after 24 hours and 40% mortality after 168 hours. On the other hand, exposure to 500 ppm of OP-EON caused 10% mortality after 48 hours and 30% mortality after 168 hours. After 48 hours, treatment with 1000 ppm of OP-EON resulted in 20% mortality, whereas treatment with 1000 ppm of OP-EON resulted in 15% mortality. Table 6 shows that the mortality rates caused by 1000 ppm of OP-EON were higher than the death rates caused by 1000 ppm of OP-EON after 72, 96, 120, 144, and 168 hours. Specifically, the mortality rates were 40%, 75%, 80%, 80%, and 85%, respectively, while the death rates were 25%, 40%, 50%, 55%, and 55%, respectively. Papanikolaou *et al.* (2022) reported that *Citrus limon* (L.) essential oil microemulsion was prepared using tween 20 caused $72.2\pm 6.0\%$ and $3.3\pm 2.4\%$ mean mortality ratio in *T. castaneum* larvae and adults after 7 days, respectively. Abdelgaleil *et al.* (2015) reported that the essential oil of *C. sinensis* exhibited significant fumigant toxicity ($LC_{50} = 19.65\text{ mg/L}$ air) and contact toxicity ($LC_{50} = 0.6\text{ mg/cm}^2$) against *T. castaneum*. Heydarzade *et al.* (2019) reported that the essential oils (EOs) of *Mentha spicata* L. and *Mentha pulegium* L., as well as their nanoemulsion forms, exhibited insecticidal activity against *T. castaneum* after 72 hours of treatment. The LC_{50} values for *Mentha spicata* L. EO and its nanoemulsion were $18.422\text{ }\mu\text{L/mL}$ and $9.279\text{ }\mu\text{L/mL}$, respectively. The LC_{50} values for *Mentha pulegium* L. EO and its nanoemulsion were $7.939\text{ }\mu\text{L/mL}$ and $6.793\text{ }\mu\text{L/mL}$, respectively. Furthermore, studies have reported that *Pimpinella anisum* L. resulted in a mortality rate of $7.33\pm 0.37\%$, while eucalyptus had an LC_{50} of $0.89\text{ }\mu\text{L/cm}^2$. Additionally, *Hazomalania voyronii* (Jum.) has also been found to have insecticidal properties. Essential oil nano-

Table 5 Mean (%) mortality of *R. dominica* after 1 h, 2 h, 4 h, 8 h, 12 h, and 16 h, and 1, 2, 3, 4, 5, 6 and 7 days in wheat exposure with 90 µL of treatment groups

Insect	Dose	Treatments	Time (hour) dependent mortality (%)									F	P	
			8	12	16	24/1d	48/2d	72/3d	96/4d	120/5d	144/6d			168/7d
<i>Rhyzopertha dominica</i>	500 ppm	Control	.00a	.00Aa	.00Aa	.00Aa	.00Aa	5.00Aab	5.00Aab	5.00Aab	10.00Ab	10.00Ab	4.70	.000
		Tween 80	.00a	.00Aa	.00Aa	.00Aa	.00Aa	.00Aa	5.00Aab	10.00Ab	10.00Ab	10.00Ab	7.50	.000
		Essential oil	.00a	5.00ABab	10.00Bb	10.00Bb	10.00Bb	25.00Bc	35.00Cd	50.00Ce	70.00Cf	75.00Cf	126.11	.000
		Essential oil nanoemulsion	.00a	10.00Bb	10.00Bb	10.00Bb	10.00Bb	20.00Bc	25.00Bc	25.00Bc	40.00Bd	50.00Be	44.38	.000
	F	-	5.50	8.00	8.00	8.00	22.66	27.00	49.00	99.00	122.75			
	P	-	.024	.009	.009	.009	.009	.000	.000	.000	.000	.000		
	1000 ppm	Control	.00Aa	.00Aa	.00Aa	.00Aa	.00Aa	.00Aa	5.00Aab	5.00Aab	10.00Ab	10.00Ab	5.87	.000
		Tween 80	.00Aa	.00Aa	.00Aa	.00Aa	.00Aa	5.00Aab	10.00Ab	10.00Ab	10.00Ab	10.00Ab	7.00	.000
		Essential oil	5.00Aa	15.00Bb	15.00Bb	60.00Cc	75.00Cd	95.00Ce	98.33Ce	98.33Ce	98.33Ce	98.33Be	426.59	.000
		Essential oil nanoemulsion	0.00Aa	10.00Bb	10.00Bb	20.00Bc	40.00Bd	50.00Be	70.00Bf	75.00Bfg	80.00Bg	90.00Bh	209.33	.000

Different lowercase letters are used to indicate horizontal differences, and different uppercase letters to indicate vertical differences. (Post hoc-Duncan test at $p \leq 0.05$). "-": statistical analysis could not be performed

Table 6 Mean (%) mortality of *T. castaneum* after 1 h, 2 h, 4 h, 8 h, 12 h, and 16 h, and 1, 2, 3, 4, 5, 6 and 7 days in wheat exposure with 90 µL of treatment groups

Insect	Dose	Treatments	Time (hour) dependent mortality (%)									F	P	
			8	12	16	24/1d	48/2d	72/3d	96/4d	120/5d	144/6d			168/7d
<i>Rhyzopertha dominica</i>	500 ppm	Control	0.00a	0.00Aa	0.00Aa	0.00Aa	0.00Aa	0.00Aa	5.00Aa	5.00Aa	5.00Aa	5.00Aa	2.25	.041
		Tween 80	.00a	.00Aa	.00Aa	.00Aa	.00Aa	.00Aa	.00Aa	.00Aa	5.00Ab	5.00Ab	2.75	.015
		Essential oil	.00a	5.00Aab	5.00Aab	10.00Bbc	10.00Bbc	15.00Bc	30.00Cad	35.00Cde	35.00Cde	40.00Ce	40.33	.000
		Essential oil nanoemulsion	.00a	.00Aa	.00Aa	.00Aa	10.00Bb	15.00Bbc	20.00Bcd	25.00Bde	25.00Bde	30.00Be	36.25	.000
	F	-	3.00	3.00	12.00	8.00	18.00	30.33	43.66	27.00	38.00			
	P	-	.095	.095	.002	.009	.001	.000	.000	.000	.000	.000		
	1000 ppm	Control	5.00Aa	5.00Aa	5.00Aa	5.00Aa	5.00Aa	5.00Aa	5.00Aa	5.00Aa	5.00Aa	5.00Aa	.75	.693
		Tween 80	.00Aa	.00Aa	.00Aa	.00Aa	.00Aa	5.00Aab	5.00Aab	10.00Abc	15.00Bc	15.00Bc	10.60	.000
		Essential oil	5.00Aa	5.00Aa	5.00Aa	15.00Bb	20.00Bb	40.00Cc	75.00Cd	80.00Cde	80.00Dde	85.00De	195.25	.000
		Essential oil nanoemulsion	.00Aa	.00Aa	.00Aa	.00Aa	15.00Bb	25.00Bc	40.00Bd	50.00Be	55.00Ce	55.00Ce	142.33	.000
F	2.00	2.00	2.00	12.00	13.33	34.75	134.75	150.75	146.75	164.00				
P	.193	.193	.193	.002	.002	.000	.000	.000	.000	.000	.000			

Different lowercase letters are used to indicate horizontal differences, and different uppercase letters to indicate vertical differences. (Post hoc-Duncan test at $p \leq 0.05$). "-": statistical analysis could not be performed

emulsion forms with significant insecticidal activity against *T. castaneum* when compared to control groups (Hashem *et al.* 2018, Adak *et al.* 2020, Kavallieratos *et al.* 2021).

The insecticidal activity of essential oils is closely related to the major components of their chemical composition. In this study, the major components of the OP-EO were found to be D-limonene (87.93%), myrcene (3.62%), and α -pinene (1.34%). According to Tripathi *et al.* (2003), d-limonene exhibited contact toxicity against adults of *R. dominica* and *T. castaneum* after 24 hours of treatment, with LD₅₀ values of 74.73 µg/mg and 79.78 µg/mg, respectively. Furthermore, it was reported that d-limonene exhibits fumigant activity against *R. dominica* and *T. castaneum*, with LC₅₀ values of 3.61 and 5.24 mg/L air, respectively. Bedini *et al.* (2015) demonstrated that myrcene,

isolated from *H. lupulus* L., exhibited the strongest repellent effect (RD₅₀ = 0.27 µM cm⁻²) against *R. dominica* for 48 hours. Abdelgaleil *et al.* (2009) reported that (-)-limonene demonstrated both contact and fumigant toxicity against *T. castaneum*, with LC₅₀ values of 494.18 µg/cm² and 33.37 mg/L, respectively, after 24 hours of treatment. Furthermore, they reported that myrcene exhibited both contact and fumigant toxicity against *T. castaneum*, with LC₅₀ values of >500 µg/cm² and 31.70 mg/L, respectively. According to Kim and Lee (2014), limonene, β -myrcene, and α -pinene exhibited contact toxicity against *T. castaneum* with LD₅₀ values of 0.283, 0.451, and 0.509 mg/adult, respectively. This toxicity was attributed to their vapor action through the respiratory system after 24 hours of treatment. In a separate study, Sun *et al.* (2020) reported

that β -myrcene and α -pinene exhibited fumigant toxicity against *T. castaneum*, with LC₅₀ values of 21.91 mg/L air and 12.15 mg/L air, respectively. Filomeno *et al.* (2020) reported that α -pinene demonstrated both contact toxicity (5.45 $\mu\text{g mg}^{-1}$) and fumigant toxicity (25.84 $\mu\text{L L}^{-1}$) against *R. dominica* after 48 hours of treatment. The mechanisms of insecticidal activity for monoterpene hydrocarbons, such as limonene, myrcene, and α -pinene, have not yet been clearly known. However, the insecticidal mode of these compounds is associated with low boiling points (140-180 °C), high volatility, inhibitory effects on acetylcholinesterase (AChE) activity, interactions with γ -aminobutyric acid (GABA), octopamine, and tyramine receptors, as well as physical effects including disruption of cell membranes and/or blocking of the tracheal system (Abdelgaleil *et al.* 2021, Gad *et al.* 2022).

The insecticidal activity of essential oils is remarkably influenced by the horizontal and vertical diffusion effects of non-polar active compounds in the insect cuticle structure. This structure includes crystalline chitin nanofibers embedded in a matrix of polyphenols, proteins, water, and lipids found in insects. In the horizontal diffusion, the active components of essential oils penetrate the tracheal system and subsequently spread to other tissues within the insect. In contrast, in the vertical diffusion, these components enter the epidermis through the tegument and then reach other tissues. However, the polarity of terpenes in essential oils significantly restricts their diffusion efficiency. The nanoemulsion forms of essential oils can show insecticidal activity through both horizontal and vertical diffusion, due to their hydrophobic, hydrophilic, amorphous surface, and negatively charged lipophilic properties (Alimoradlu and Zamani 2022). The essential oils nanoemulsions have a large surface area, which can suggest advantages in terms of contact toxicity by extending the insect contact area and duration (Margulis-Goshen and Magdassi 2013, González *et al.* 2014, Osanloo *et al.* 2017, Patino-Portela *et al.* 2021, Azmy 2021). The insecticidal activity of nanoemulsions is based on various mechanisms, including the induction of oxidative stress in cells, inhibition of biochemical pathways, and interaction and denaturation of proteins and DNA, ultimately resulting in cell death (Benelli 2018, Azmy *et al.* 2019, Sharma *et al.* 2020). The OP-EON used in this study contained 4% (v/v) OP-EO. Thus, the actual concentration of EO in EO-N solutions (at 500 and 1000 ppm) was 25-fold lower than the concentration of pure EO tested at the same levels. This explanation highlights the variations in mortality rates and demonstrates that the insecticidal efficacy succeeded with the OP-EON, developed to provide the sustainability of the OP-EO, holds significant potential. The OP-EON produced in this study showed more potent insecticidal activity against *R. dominica* and *T. castaneum* compared to the OP-EO.

4. Conclusions

In this study, a nanoemulsion formulation was developed and characterized to improve the insecticidal activity of OP-EO. The effectiveness of the nanoemulsion formulation against *R. dominica* and *T. castaneum* was also

investigated. The OP-EON (contains 4% (v/v) EO) exhibited significantly higher insecticidal activity against *R. dominica* and *T. castaneum* compared to the OP-EO (contains 100% (v/v) EO). The insecticidal activities of OP-EO and OP-EON were more effective against *R. dominica* than *T. castaneum*. The dose and treatment durations of the treatment groups played a crucial role in their insecticidal activities. While the study shows promising results regarding the high insecticidal activity of OP-EON, further research is necessary to assess its potential impact on human health, non-target organisms, and environmental cycles.

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