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Enhanced repellent and anti-nutritional activities of polymeric nanoparticles containing essential oils against red flour beetle, *Tribolium castaneum*

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Encapsulation of essential oils (EOs) is an important strategy that can be applied to intensify the stability and efficiency of these compounds in integrated pest management. The present study aimed to investigate the sub-lethal activity of polymer-based EOs nanoparticles against red flour beetle, *Tribolium castaneum* adults as an important critical pest of stored products. Chitosan nanoparticles (CSNPs) containing garlic and cinnamon essential oils (GEO and CEO) prepared using the ionic cross-link technique. Stability of nano-formulations evaluated over temperature and storage time. The fumigant effect (LC_{10}, LC_{20}, LC_{30}) and contact toxicity (LC_{10}, LC_{15}, LC_{25}) determined. In addition, the contact toxicities of EOs and their nanoparticles on nutritional indices evaluated. An olfactometer used to assess the repellent activity of EOs and EOs loaded in CSNPs (EOs@CSNPs) in sub-lethal fumigant concentrations. Characterization results showed GEO loaded in CSNPs has particle size of 231.14 ± 7.55 nm, polydispersity index (PDI) value of 0.15 ± 0.02, encapsulation efficiency (EE) percentage of 76.77 ± 0.20 and zeta potential of − 18.82 ± 0.90 mV, in which these values for the CEO loaded in CSNPs (CEO@CSNPs) changed to 303.46 ± 0.00 nm, 0.20 ± 0.05, 86.81 ± 0.00% and − 20.16 ± 0.35 mV, respectively. A lower PDI value for both CSNPs showed an appropriate NPs size distribution. Furthermore, NPs size and encapsulation efficiency did not change in various temperatures and during four months which confirm good stability of the EOs@CSNPs. In LC_{30} of GEO@CSNPs, the maximum repellency was determined as 66.66 ± 3.33. Among nutritional indices, in LC_{25} of GEO@CSNPs, the relative growth rate (RGR) (0.011 ± 0.003 $mg.mg^{-1}.day^{-1}$), relative consumption rate (RCR) (0.075 ± 0.004 $mg.mg^{-1}.day^{-1}$) and feeding deterrence index (FDI) (54.662 ± 1.616%) were more affected, so GEO@CSNPs was more effective than CEO@CSNPs. The results of repellent and anti-dietary activities of EOs and EOs@CSNPs confirmed the higher repellency and adverse effectivity on nutritional indices of *Tribolium castaneum* pest treated with EOs@CSNPs compared to free EOs. In conclusion, the NPs form of GEO and CEO can be a novel and efficient carrier for improving the repellent and anti-nutritional activities of EOs.

Keywords Pest management, Garlic essential oil, Cinnamon essential oil, Chitosan polymeric nanoparticles, Repellency, Nutritional indices

Abbreviations

AFM	Atomic force microscope
CS	Chitosan
CSNPs	Chitosan nanoparticles
CEO	Cinnamon essential oil
CEO@CSNPs	Cinnamon essential oil loaded in CSNPs

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DD	Degree of deacetylation
ECI	Efficacy of conversion of ingested food
EE	Encapsulation efficiency
EOs	Essential oils
FDI	Feeding deterrence index
FTIR	Fourier transform infrared spectroscopic
GEO	Garlic essential oil
GEO@CSNPs	Garlic essential oil loaded in CSNPs
GC-MS	Gas Chromatograph mass
PDI	Polydispersity index
RCR	Relative consumption rate
RGR	Relative growth rate
RI	Repellent index
TEM	Transmission electron microscopy
TPP	Tripolyphosphate

Nowadays, the quality and quantity of cereal products have been reduced as a result of insect infestation. It is one of the major issues for publics, food industries, and health organizations[1]. The red flour beetle, *Tribolium castaneum (T. castaneum),* a secondary polyphagous pest, has been reported commonly from 246 grain and processed stored products so far, especially flour and starchy products worldwide[2]. Besides quantity damage of products obtained from *T. castaneum* nutritional activities, the quality damage observed through their rapid population growth followed by uric acids being in larval feces, strings, excrements, cover change skins and dead insect debris[3]. In addition, the activity of various pests such as *T. castaneum*[4–6], *Sitophilus zeamais*[7], *Tribolium confusum, Attagenus megatoma*[8] increased the storage medium temperature, humidity and ultimately rapid growth of toxic fungi such as *Aspergillus flavus*[6], *Penicilium*, *fusarium*, and etc.[5,9]. Moreover, the carcinogenic compounds such as 1,4-benzoquinone, methyl-1,4-benzoquinone and ethyl-1,4-benzoquinone produced by *T. castaneum* seems to be as an external defensive secretion. Therefore, these constituents removed microbes and inhibited the predator's activity plus self-regulated population growth[2,10]. In addition, these compounds, plus uric acid, caused toxic effectivity of stored foods. The chemical insecticides such as organophosphates, pyrethroids and fumigants were the most common and low cost-effective methods for controlling storage insects[11]. However, chemical insecticides have numerous problems such as prevent crops and human health protection, besides polluted the environment. Applying components based on plants such as essential oils (EOs) is a suitable way in integrated pest management[12–14]. EOs significantly considered in many researches due to their multiple properties such as antibacterial, antimicrobial, antiplasmodial, antifungal, anticancer and insecticidal[15–18]. EOs are volatile and lipophilic complexes produced by plants that decompose rapidly in nature[19]. The secondary metabolites of plants are very different. After being metabolized, they produce various products such as phenols, terpenes, sesquiterpenes, hydrocarbons, hydroxyls, carbonyl groups, oxygenated groups, sulfurs, nitrogen, and mineral compounds. These products are affected by climate, soil, and geographical region which can synergistically or separately control insects. However, it is not possible to determine which of these compounds play the main role against herbivorous insects and pathogens[20]. These compounds have different and complex mechanism of action due to their complex structure and different metabolites[21–25]. Different effects of EO formulations including repellency, feeding deterrent, fecundity, fertility, reproduction, growth rate and life time of different pests have been studied[26,27]. Despite the beneficial properties of EOs, rapid oxidation and poor solubility in water have caused a short-term effectiveness[28] compared to synthetic chemicals. Applying stabilizers or changing the common form of EOs to nano-formulations, can improve EOs properties[24,29]. Among various materials for essential oil encapsulations, polymeric nanoparticles (NPs) are one of the promising emerging methods. Encapsulation produced by a uniform coating or mixture of polymers surrounded with active materials to improve the stability, longevity and accessibility to main materials easily. Polymers used for formulations of insecticides based on EOs include various polysaccharides (such as chitosan (CS), alginates, starch) and polyesters (such as poly £ -caprolactone, polyethylene glycol (PEG))[30]. The polymer selection was based on various parameters such as application, safety, compatibility and biodegradation, low cost, and availability[29,31]. Among different polymers, CS with a hydrophilic and polycationic nature selected that considered as a nano carrier with pH-sensitivity property that provided a control released of biological components[32]. The acute fumigant and contact toxicity of nano-pesticides have been studied plenty on different groups of insects[29,33]. However, limited studies conducted on sub-lethal effects of nano-pesticides for controlling pest insects. Sub-lethal effects defined as organisms effectivity that survive after exposure to a pesticide[34]. The sub-lethal effects can reduce the reproduction (resulting from impaired gametogenesis, potential fertility, and mating patterns), life times, developmental and metabolic disorders, repellency and nutritional activities[29,35]. Insect repellents are materials usually act locally or remotely to prevent arthropods from flying toward landing, biting humans and animals and contact surfaces in general by creating a vapor barrier[36,37]. In 2021, Kumar and Hemalatha prepared PEG–cardamom EO@NPs with significant repellent effect against adult insects like *Oryzaephilus surinamensis* and *Elettaria cardamomum*[38]. In another study, nano-emulsions of *Baccharis reticularia* and its constituents exhibited repellent activity against *T. castaneum* remarkably in constituents as limonene and α-pinene nano-emulsions[39]. González et al. (2014) showed that bergamot and geranium NPs can affect the nutritional physiology of two pests, *T. castaneum and Rhizopertha dominica*[40]. In addition, they examined the repellent enhancement of these two nano-formulations on another pest, *Blattella gennanica* with negatively effectiveness on the nutritional indices[29]. Heydarzade et al., observed that *Mentha spicata* and *Mentha pulegium* EO nano- formulations altered nutritional indices[41].

In the present study, polymeric NPs containing GEO and CEO prepared using ionic gelation technique to achieve the stable, small, and uniform chitosan nanoparticles (CSNPs) with an acceptable entrapment efficiency (EE) of EOs. Then, the efficacy of the prepared CSNPs was evaluated. This is the first study that examine the CSNPs efficacy on the sub-lethal effects including repellency and anti-nutritional indices against *T. castaneum* as a secondary polyphagous pest. Finally, sub-lethal effects of nano-formulations compared with pure EOs.

Materials and methods

Materials

Sodium tripolyphospate (TPP), Deacetylated CS (medium molecular weight 190–310 kDa, 87% degree of deacetylation (DD)) and glacial acetic acid provided from Sigma- Aldrich Company (USA). Sodium hydroxide and Tween 80 bought from Merck Company that acts as pH modulator and emulsifier, respectively. Chloroform purchased from Carlo Erba Company and used as a solvent. Cinnamon sticks and Hamadan garlic cloves were obtained from the local markets with confirmed scientific names by *botanists of Ferdowsi University* as *Allium sativum* and *Cinnamomum cassia,* respectively.

Extraction of *EOs*

To obtain EOs from natural plant raw materials, garlic cloves were dried and powdered with an electric grinder. Dried cinnamon sticks are also crumbled. EOs extraction was conducted by hydrodistillation using Clevenger apparatus after 3 h at the temperature of 120 °C. The extracted EOs were dehydrated by anhydrous sodium sulfate and kept in a dark glass container in a refrigerator at 4 °C until use[42]. The percentage of the extracted EOs yield was calculated by Eq. 1[43]:

$$\text{EOs yield } (\%) = \frac{\text{v(ml)}}{\text{m(g)}} \times 100 \quad (1)$$

where v is the volume of the EO and m is the weight of the sample (g).

Preparation of CEO and GEO loaded in CSNPs

To encapsulate GEO and CEO into polymeric nanoparticles, ionic gelation method[44,45] with a slight modification used by applying CS (natural polymer) and TPP (Crosslinker). Briefly, CS (3 mg/ml) was dissolved in glacial acetic acid at 1% V/V. Then, solution stirred 24h for homogenization. The pH of the obtained solution was adjusted to 4.5–5 using NaOH 1 M. TPP and Tween 80 solutions separately prepared with adding 0.1 g of TPP or Tween 80 to 10 ml of deionized water. 0.2 ml of TPP and 8 ml of Tween 80 mixed. Then, CEO by dissolving tendency in organic compounds was dissolved in chloroform (175 mg/ml). 0.2 ml of CEO solution added to TPP and Tween 80 solution and agitated for 15 min. Finally, CSNPs were formed by dropwise adding of the final solution to 5 ml of CS solution under magnetic stirring (500 rpm) for 3h. The final suspension was ultrasound with a probe at amplitude 80 W and cycle 0.5 for 15 min. The ice bath used to avoid temperature increase. Then, it was centrifuged at 10,000 rpm for 30 min at 4 °C The same method for encapsulation of GEO in CSNPs used.

To investigate the impact of CS: TPP mass ratio, different samples prepared with 2, 5, 7.5 and 10 CS: TPP mass ratios. Among them, the optimum sample based on smaller and uniform NP size and higher EE selected. The blank CSNPs prepared similarly without EOs.

Gas chromatograph mass (GC–MS) chemical characterization

After extraction, EOs components were determined by GC-MS (HR-GC 5000, Konik, Spain)[1,46]. The experimental conditions for GC-MS analysis described in Table 1. In addition, to calculate EE percentage of EOs@CSNPs, GC-MS was applied.

CSNPs Characterizations

The hydrodynamic particle size, PDI value and zeta potential of CSNPs measured by particle size analyzer (Vasco3-Cordouen, France) in ambient temperature.

The CSNPs morphology investigated by transmission electron microscopy (TEM) (EM120, Philips, Netherland) and atomic force microscope (AFM)(Arapazhoohesh, Iran) analysis. Also, the chemical bonds of

	Essential oil	
	Cinnamon	**Garlic**
Column	HB-5NS column (30 m × 0.25 mm × 0.25µm film thickness)	HB-5NS column (30 m × 0.25 mm × 0.25µm film thickness)
Carrier gas (flow rate)	Helium (1 ml.min^{-1})	Helium (1 ml.min^{-1})
Injection volume	1 µl	1 µl
Temperature programming	Injector temperature: 209 °C	Injector temperature: 220 °C;
	Column temperature: started from 20 °C maintained for16 min, and ended at 221 °C	Column temperature: started from 40 °C maintained for 2 min, and ended at 240 °C maintained at 15 min
Ion source temperature	201 °C	240 °C
Ionization mode	Electron impact (71eV)	Electron impact (70eV)

Table 1. Experimental conditions of the GC–MS analysis for cinnamon and garlic essential oils.

constituents were identified and detected by Fourier transform infrared spectroscopic (FTIR)(Thermo Nicolet, USA).

The free EOs value in supernatant measured by GC-MS after 15 min suspension centrifugation at 4°C (10000 rpm). The EE% calculated by the mentioned equation[33]:

$$EE\% = \frac{Total\ amount\ of\ oil - Free\ amount\ of\ oil\ in\ supernatant}{Total\ amount\ of\ oil} \times 100 \quad (2)$$

Rearing insects

The first colony of *T. castaneum* was isolated from insect laboratory of Ferdowsi University of Mashhad, Iran. Insects were raised in a 282.6 cm^3 glass container (height 10 cm × diameter 6 cm) on wheat flour mixed with yeast (10:1, w/w) with 100 unsexed adults. After 3 days, adults removed, and the cultures conserved in a growth chamber adjusted with 27 ± 1 °C and 60 ± 5% relative humidity. For all tests, the 2–7 days old unsexed adults were used[4].

Preparation of flour discs

The flour disc chips prepared with 1.3 cm diameter. Each flour disc composed of aliquots (200 µl) of flour suspension (10 g flour in 50 ml distilled water) and putting them onto the surface of plastic Petri dishes and kept them at the temperature of 27°C and 60–70% RH during 12 h for drying[29].

Repellency evaluation

A Y-shaped olfactometer used to evaluate the EOs and their nano-formulations repellent activities as described by Daniel[47]. This device is composed of a main arm (20 cm length) with two accessorial arms (15 cm length) at the end. The angle between accessorial arms was 90°, and the inside diameter of arms was 2 cm. The olfactometer volume was 75 ml. One of accessorial arms treated with EOs or their nano-formulations, so another arm treated as control. Different series of CSNPs formulations without EOs were diluted by distilled water. One of the advantages of loading essential oils in nanoparticles is increasing their solubility in water, while essential oils alone do not dissolve in water due to their hydrophobic properties. Chloroform was applied to solve EOs. However, the non-toxicity of chloroform on the adult red flour beetles will be investigated. The flour discs treated with an aliquot of 10 µl from each concentration. Fumigant bioassays were conducted based on odour essential oils like other studies[47]. LC_{10}, LC_{20} and LC_{30} were calculated from fumigant bioassays. Narrow concentrations with 20–80% mortality were chosen between 0.29–1.2/0.34–0.8 $\mu l.l^{-1}$air for GEO and GEO@CSNPs and 0.25–2.8/0.5–1 $\mu l.l^{-1}$air for CEO and CEO@CSNPs, respectively[33]. However, concentrations applied for CEO and its nano-formulation were 0.018, 0.03, 0.041 $\mu l.l^{-1}$. For GEO and its nano-formulation, other concentrations including 0.021, 0.028, 0.035 $\mu l.l^{-1}$ used which were equivalent to LC_{10}, LC_{20} and LC_{30} of fumigant bioassays, respectively. The fumigant bioassays were done in dark glass containers (1004.8 cm^3)[33]. The assessment performed on adult red flour beetles. After using chloroform as a solvent, the time needed for its evaporation was 20 min. Then, an adult of *T. castaneum* isolated from the insect colony, kept starving for 24 h, and released into the entrance chamber. All experiments were conducted at 27 ± 2 °C and 60 ± 5%RH in the dark incubator. After 24 h, the insect positioning was recorded (60 insects released). A procedure as group repellency also used in which, for each concentration, 20 insects released in 3 replications. Repellent activity determined as a percentage with the formula (Eq. 3):

$$\text{Repellent Index (RI\%)} = \frac{C - T}{G} \times 100 \quad (3)$$

In which, C, T, and G are referred to the number of insects in the control, treatment, and total number of insects, respectively[39]. RI values higher than 0.1, lower than 0.1 and between them indicated that the EOs and their nano-formulations were repellent, attractant, and neutral, respectively[48].

Investigation of nutritional indices

Anti-nutritional effects of EOs and their nano-formulations investigated based on the procedure explained by González et al.[29] with slight modfications. In this test, each disc treated with 10 µl aliquots of each concentration and allowed for oil evaporation for 15 min. The control treated with chloroform or CSNPs oil-free solution. Three concentrations of GEO prepared as 0.020, 0.0228, and 0.0274 $\mu l.disc^{-1}$, and those of CEO as 0.0095, 0.0108, and 0.0130 $\mu l.disc^{-1}$. These concentrations were equal to LC_{10}, LC_{15}, and LC_{25} contact toxicities of EOs and EOs@CSNPs. The contact toxicities were done by 9 cm diameter Petri dish with the Whatman filter paper (NO. 1) that was placed at the bottom of Petri dish[33]. Ten 24 h-starved *T. castaneum* weighed and transferred to each Petri and maintained in the controlled growth chamber at 27 ± 1 °C with 65 ± 5% relative humidity and darkness. Five replications used. After 3 days of treatment, the weights of discs and alive insects determined. Nutritional indices determined using the following equations (Eqs: 4–7)[40]:

$$\text{Relative growth rate index (RGR)}(mg.mg^{-1}.day^{-1}) = \frac{A - B}{B \times day} \quad (4)$$

$$\text{Relative consumption rate (RCR)}(mg.mg^{-1}.day^{-1}) = \frac{D}{day \times B} \quad (5)$$

$$\text{Efficacy of conversion of ingested food (ECI\%)} = \frac{RGR}{RCR} \times 100 \quad (6)$$

$$\text{Feeding deterrence index (FDI\%)} = \frac{C - T}{C} \times 100 \quad (7)$$

In RGR equation, A includes the ultimate weight of alive insects divided by the number of alive insects after bioassay tests (3 days), and B is the main weight of insects divided by the total number of insects.

RCR shows that the consumption of insects is affected by the two factors including initial weight and the duration of the experiment. In the mentioned equation, D represents the consumption of biomass divided by the number of alive insects after conducting experiments.

ECI indices indicates the amount of food consumed by insect to achieve more weight. In the FDI equation, C and T are the amount of insect consumption from control and treatment discs, respectively. Positive and negative values indicated feeding deterrence and stimulant effects, respectively.

Statistical analysis

It is necessary to do sub-lethal bioassays with alive insects. So, it is common to use ($LC < LC_{50}$). So, bioassays analysis at lower LC such as LC_{10}, LC_{15}, $LC_{20,}$ LC_{25} and LC_{30} can be applied as mentioned in similar studies[29,41,49–51]. To investigate the repellency and anti-nutritional indices of insects, the sub-lethal LC values of fumigant and contact toxicities were obtained as described as previous study by Probit Analysis (1972) using POLO-PC software[33]. The repellency effect of EOs and EOs@CSNPs and the nutritional data were analyzed using One-Way ANOVA and Tukey's HSD. Statistical analysis conducted by the SPSS V. 16 program.

Results and discussion

The yield of the extracted GEO and CEO was 0.26 ± 0.01% v/w and 0.62 ± 0.045% v/w, respectively. Results of GC–MS analysis of extracted EOs are shown in Fig. 1a and 1b. For CEO, cinnamaldehyde with relative values 87.2%, and for GEO, allyl trisulfide plus diallyl disulfide with values 45.5% and 21.3%, respectively were identified as abundant ingredients.

GC–MS analysis results identified the necessary and abundant components in GEO as allyl trisulfide, diallyl disulfide, and methyl allyl trisulfide followed by relative values of 45.5, 21.3, and 19.2%. Abundant components for CEO were Cinnamaldehyde, (E)-(87.2%), α-Copaene (3.8%), o-Methoxycinnamaldehyde (2.4%) and Cadina-1(6),4-diene (2%). Our findings are in agreement with the previous studies for GEO[52–54] and CEO[1,55,56]. Different types and amounts of EOs ingredients can be affected by environmental factors like temperature, soil composition, climatic conditions, environmental stress, ecosystem, altitude, method of oil extraction, and plant genetics[57,58].

The GEO@CSNPs presented the hydrodynamics size of 231.14 ± 7.55 nm with PDI value of 0.15 ± 0.02, zeta potential of – 18.82 ± 0.90 mV, and EE% of 76.78 ± 0.20%. For CEO@CSNPs, the size, PDI value, zeta potential, and EE% recorded as 303.46 ± 0.01 nm, 0.20 ± 0.05, -20.16 ± 0.35 and 86.81 ± 0.01%, respectively. The size of CEO@CSNPs and GEO@CSNPs estimated at 27 and 22 nm based on TEM analysis (Fig. 2a, b). Higher encapsulation of CEO in CSNPs produces larger NPs than GEO@CSNPs. This is due to the presence of cinnamaldehyde with lower molecular weight in CEO@CSNPs compared to other components of GEO@CSNPs, such as diallyl disulfide and allyl trisulfide with higher molecular weight[45]. Furthermore, TEM size of NPs was lower than the hydrodynamic size (Fig. 2c, d). Dynamic light scattering (DLS) analysis shows the hydrodynamic size while the actual size of nanoparticles based on TEM analysis is lower than DLS sizes. 2D and 3D images of AFM analysis (Fig. 2e, 2f) confirmed the uniform distribution of nanoparticles in both nano-formulations which is in agreement with TEM analysis. Different factors such as the concentration and molecular weight of CS, the degree of acetylating, the ratio of materials used, the mixing method, the pH of the system, and their interactions can affect the NPs size[59–62]. As shown in TEM image, CSNPs had a spherical appearance. The spherical appearance of the nanoparticles enhances controlled release and pesticide protection, which may be due to the longer path time of the EOs through nanoparticles than other forms[63]. In addition, the mobility of nanoparticles is more than pure EOs, therefore they can penetrate insects' tissue, quickly[64]. In the other case, when NPs held in the extracellular area, the percentage of detoxification decreased. Therefore, they are not available for detoxifying systems; thus, further bioactive compounds reach the action-site, improving the toxic influences of pure oil[14].

According to Servat-Medina et al. study, the particle size of CSNPs loaded with *Arrabidaea chica* was 150 nm up to 681 nm. They realized that the lowest NP size (150 ± 13 nm) obtained for CS: TPP mass ratio of 5 among various mass ratios of 20, 10, 7, 5, and 4[65].

The zeta potentials as suspension stability were in the range of -18 up to -21 that are acceptable. One of the reasons for negative zeta potential may be due to the surfactant presence on the surface of the NPs. Furthermore, the presence of EOs with negatively charged molecules such as diallyl disulfide (zeta potential of – 31 mV)[66] on the surface of CSNPs may produce negative zeta potential of NPs.

The obtained EE% for both EOs was higher than 70%. The EE% slightly reduced after 4 months of storage time based on GC–MS analysis. However, this reduction was not significant ($P > 0.05$). Hence, CSNPs are suitable carriers for both EOs that protect volatile components and lead to controlled release of components. Su et al. reported 4.6–32.9% EE for CSNPs loaded with CEO[67]. Also, Gupta et al. mentioned 60–75% EE for CSNPs loaded with garlic aqueous extract[68].

One of the important parameters in designing drug carrier is controlling particle size distribution in a narrow domain with high stability and lower size enhancement during storage time. The stability of CEO@CSNPs and GEO@CSNPs examined based on EE% after production, 2 and 4 months (Table 2) at the storage condition of 4

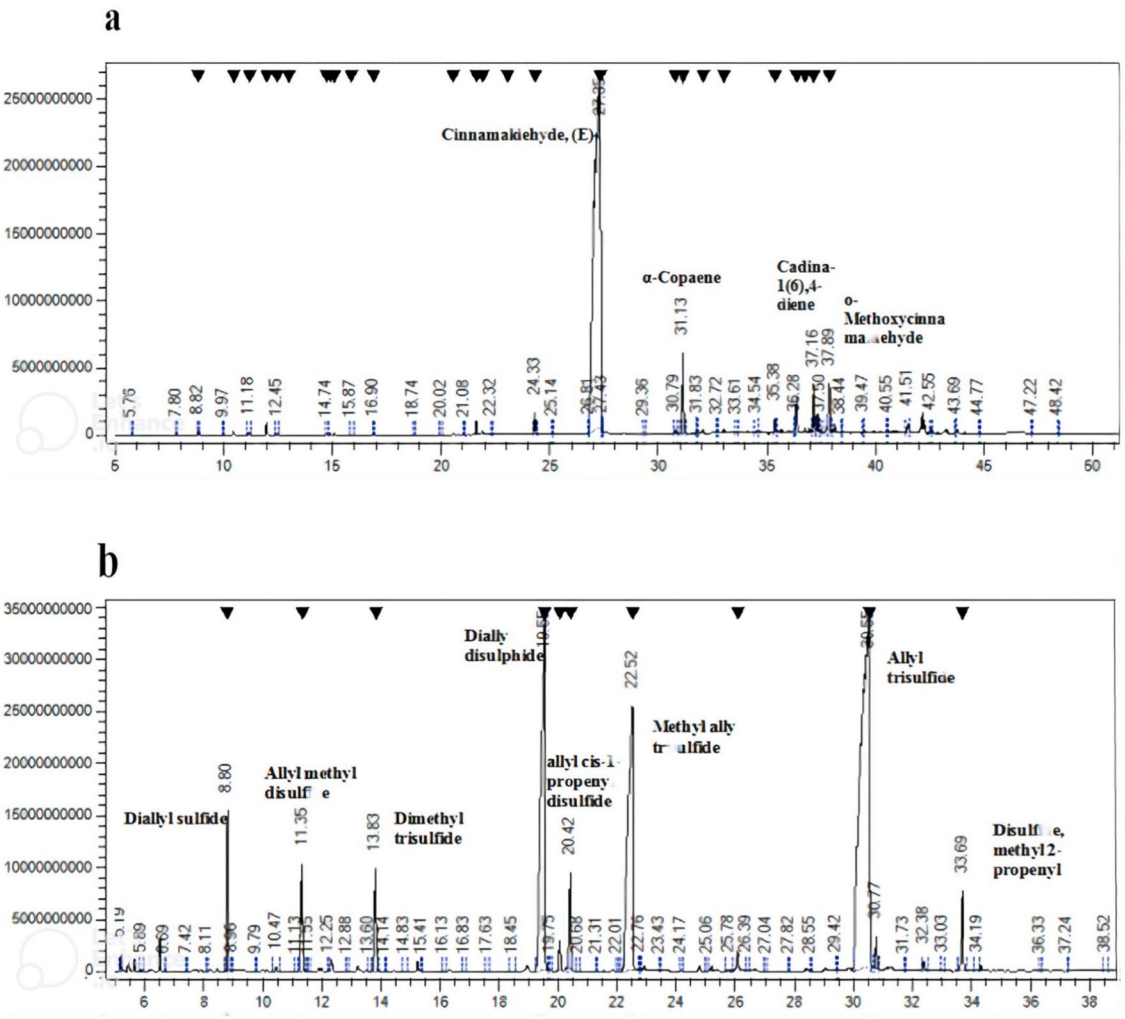

Figure 1. GC–MS analysis of abundant compounds in (**a**) CEO and (**b**) GEO.

°C, 25 °C, and 40 °C. As Table 2 shows there was a slight reduction in EE% values after four months. Based on the one-way ANOVA, the calculated P-values for size and EE% versus various times were greater than 0.05, so size enhancement and EE% reduction were not significant. This approves high stability of prepared NPs during storage time. The FTIR spectra of CS, CEO, GEO, CEO@CSNPs, and GEO@CSNPs are shown in Fig. 3. In the FTIR spectrum of CS powder, a broad peak at the limited area of 3400 to 3700 cm^{-1} attributed to the combination of O–H and N–H tensile vibration bonds. The assigned peaks in 1158.82, and 1261.21 cm^{-1} are allocated to C–O–C asymmetric tensile and O–H bonds, respectively. Another peak observed in 604.46 cm^{-1} related to the pyranose ring tensile vibrations. Also, specific peaks at 1654, 1600, and 1383 cm^{-1} are allocated to amid I, II, and III, respectively[45,69–72].

For CEO, various peaks for CEO at 689 and 748 cm^{-1} is related to the C–H tensile of benzene and alkene ring, respectively[73]. The peak at 1575cm^{-1} was belong to the C-O tensile of aromatic ring[74], and another peak at 2923 cm^{-1} was related to the C-H tensile bond[67].

In the FTIR spectrum of CEO@CSNPs, the peaks related to the CS amide groups (1654, 1600, and 1383 cm^{-1}) transferred to 1576, 1631 and 1466 cm^{-1}. The emerging new peak at 1738 cm^{-1} confirmed the cross-linking between TPP phosphate and CS amino groups[33].

According to the CEO and CSNPs signed overlap peaks, following peaks such as 688, 748, and 1575 cm^{-1} can be considered as CEO characteristic peaks with a lower height, that pointed in the FTIR spectrum of CEO and CSNPs that confirmed the CEO entrapment in the CSNPs.

For GEO, different peaks reported in 923 cm^{-1} (N–H tensile vibration of proteins), 1077 cm^{-1} (SO_3 symmetric tensile vibration), 1306 cm^{-1} (C–O tensile vibration of aromatic ester), 1424 cm^{-1} (C–H tensile vibration especially in lipids), and 2977 cm^{-1} (C–H tensile especially in lipids)[75,76]. In the FTIR spectrum of GEO@CSNPs, the related peaks to the CS amide group (1654 cm^{-1}, 1600 cm^{-1}, 1383 cm^{-1}) changed to the new peaks at 1629, 1575, and 1339 cm^{-1}, respectively that confirmed the cross-linking between TPP and CS amino groups. Some peaks of GEO spectrum at 923, 1306, and 2977 cm^{-1} has been pointed in GEO@CSNPs spectrum with reduced height that informed the successful encapsulation of GEO in CSNPs.

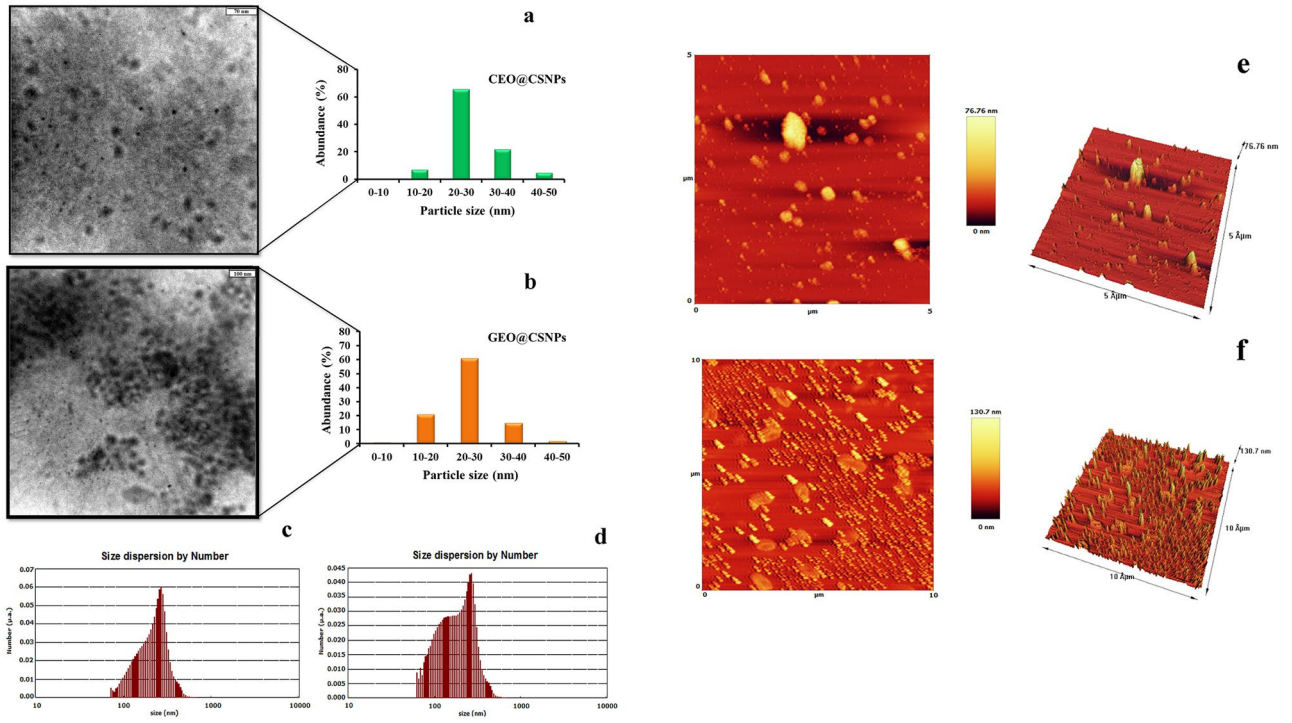

Figure 2. TEM images of (**a**) CEO@CSNPs, (**b**) GEO@CSNPs (dark spheres on the images are CSNPs), DLS size distribution of (**c**) CEO@CSNPs and (**d**) GEO@CSNPs, AFM images at both 2D and 3D view for (**e**) CEO@CSNPs and (**f**) GEO@CSNPs.

Characteristic	Storage time ψ	EOs@CSNPs					
		CEO@CSNPs mean (%) ± SE			GEO@CSNPs mean (%) ± SE		
		4 °C	25 °C	40 °C	4 °C	25 °C	40 °C
EE (%)	0	86.81 ± 0.01	83.01 ± 0.30	79.86 ± 0.80	76.78 ± 0.21	73.48 ± 0.13	69.84 ± 0.37
	2	85.12 ± 0.80	82.07 ± 0.45	78.27 ± 1.85	75.88 ± 0.60	72.37 ± 0.14	67.91 ± 1.69
	4	84.24 ± 0.28	80.84 ± 0.61	76.99 ± 1.61	74.86 ± 0.48	70.21 ± 1.06	64.60 ± 1.03
Size (nm)	0	303.46 ± 0.02	305.95 ± 0.6	308.56 ± 0.33	231.14 ± 7.55	234.91 ± 7.24	238.36 ± 6.9
	2	328.135 ± 19.75	331.54 ± 19.70	335.00 ± 19.36	268.525 ± 18.86	271.12 ± 18.1	273.82 ± 17.41
	4	337.61 ± 30.97	340.56 ± 29.85	343.26 ± 29.16	280.72 ± 26.49	283.21 ± 25.89	285.82 ± 25.62

Table 2. Encapsulation efficiency and size of EOs@CSNPs over storage time. Ψ Regarded as the time after preparation of nano-formulation. Based on the one-way ANOVA analysis, the calculated P-value for EE(%) was higher than 0.05 for 4 °C, 25 °C and 40 °C between various times, so EE% decrease was not significant in 4 °C, 25 °C and 40 °C Based on the one-way ANOVA analysis, the calculated P-value for size (nm) at different temperatures during 4 month was higher than 0.05, so size change was not significant.

The LC_{10}, LC_{15}, LC_{20}, LC_{25}, LC_{30}, and LC_{50} values for EOs and their CSNPs on *T. castaneum* adults are reported in Table 3. No control mortality was observed during the study. The slope of the log-dose Probit relationship (homogeneity of response) was also reported. A large (>2) and small slopes (<2) indicates a homogenous and heterogeneous populations, respectively[29]. The slopes in this study were higher than 2, no significant differences were observed.

To evaluate the repellent effects of EOs, CEO@CSNPs, and GEO@CSNPs, an olfactometer apparatus was used. The system currently attracted or repellent according to the odors of EOs or their nano-formulations. The percentage RI is the time spent by each beetle toward the treated and non-treated regions of olfactometer. According to the RI values after 24h, EOs and their nano-formulations presented repellent activity of RI > 0.1. Table 4 shows the RI values for the EOs and EOs@CSNPs. All treatments had significant repellency during 24h exposure at the LC_{30} values (P-value < 0.05). Between two EOs, GEO had more repellent effect than CEO, due to the higher values of RI during 24h. Similar trend was observed for EOs@CSNPs. EOs nano-formulations increased the repellent effects; so that the CSNPs enhanced the effectivity of EOs. During all the experiments, GEO@CSNPs were more effective than CEO@CSNPs, indicated the highest RI values. The repellent efficiency increased as the concentration was increased. At LC_{30} concentrations of the CEO@CSNPS and GEO@CSNPs, the

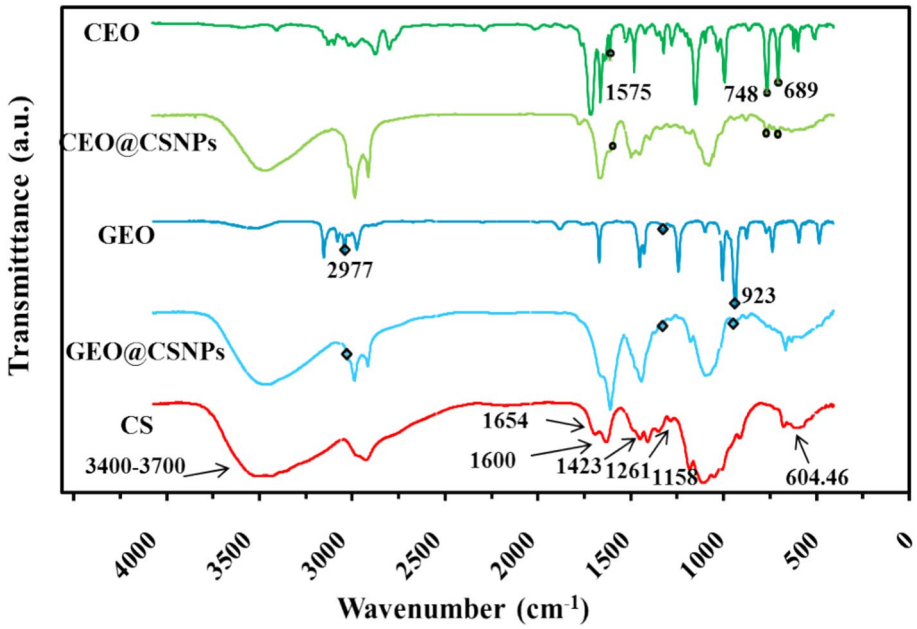

Figure 3. FTIR spectra of CS powder, GEO, CEO, GEO@CSNPs, and CEO@CSNPs.

			Sub-lethal concentrations				Lethal concentration			
Treatment	**N**	**Bioassay tests**	**LC_{10}**	**LC_{15}**	**LC_{20}**	**LC_{25}**	**LC_{30}**	**LC_{50}**	**Slope (±SE)**	**X^2 (df)**
CEO	700	fumigant(μll^{-1} air)	0.25(0.20–0.31)	–	0.40(0.34–0.46)	–	0.55(0.49–0.62)	0.94(0.86–1.06)	2.26(±0.17)	0.88(4)
		Contact(μlm^{-2})	149.41(133.68–165.13)	169.85(154.12–185.58)	–	204.45(190.30–220.18)	–	290.95(276.79–308.25)	4.45(±0.34)	0.064(4)
CEO@CSNP	700	fumigant(μll^{-1} air)	0.47(0.44–0.50)	–	0.54(0.51–0.57)	–	0.59(0.57–0.62)	0.70(0.68–0.72)	7.52(±0.59)	0.52(4)
		Contact(μlm^{-2})	75.49(69.20–83.35)	84.93(77.06–91.22)	–	99.08(92.79–105.37)	–	135.25(128.96–143.12)	5.08(±0.37)	0.76(4)
GEO	700	fumigant(μll^{-1} air)	0.29(0.26–0.33)	–	0.38(0.35–0.42)	–	0.47(0.43–0.50)	0.64(0.61–0.69)	3.78(±0.29)	1.85(4)
		Contact(μlm^{-2})	317.68(286.23–349.14)	358.58(328.69–390.03)	–	430.92(402.61–460.80)	–	605.49(577.18–641.66)	4.57(±0.35)	2.38(4)
GEO@CSNP	700	fumigant(μll^{-1} air)	0.33(0.31–0.35)	–	0.38(0.36–0.41)	–	0.43(0.41–0.45)	0.51(0.50–0.53)	6.80(±0.51)	1.44(4)
		Contact(μlm^{-2})	124.24(108.52–139.97)	144.69(128.96–160.42)	–	180.86(166.71–198.16)	–	278.37(261.07–298.81)	3.66(±0.28)	0.98(4)

Table 3. LC_{10}, LC_{15}, LC_{25}, LC_{30}, and LC_{50} achieved with data mortality of fumigant and contact toxicity of pure oils and CSNPs loaded oils against *T. castaneum* adults after 24h. The 95% lower and upper confidence intervals are reported in parentheses, respectively.

repellent activity of adults was promoted to 56.67% and 66.6%, respectively. The lowest repellent activity (23.33%) was observed at LC_{10} (0.018 μl.l^{-1}) of CEO, reaching 66.66% at LC_{30} (0.032 μl.l^{-1}) of GEO@CSNPs (Table 4).

The nutritional indices of *T. castaneum* adults fed on various concentrations of pure EOs and their nano formulation counterparts are represented in Table 5.

The statistical analysis of the nutritional indices and the anti-feeding activity showed that at LC_{25} concentration, GEO@CSNPs significantly decreased the RGR, RCR and ECI ($P<0.05$). Moreover, at two concentrations (LC_{15} and LC_{25}), significant differences were seen in FDI between GEO@CSNPs and pure EOs (P-value <0.05). At LC_{10} concentration, just RGR and RCR were affected by EO@CSNPs (P-value <0.05); no differences were observed in the other studied parameters including FDI (P-value>0.05) and ECI (P-value>0.05). At LC_{15} concentration, RGR, RCR, and FDI were affected by EOs@CSNPs (P-value<0.05) except ECI (P-value >0.05)(Table 5).

By increasing the concentration of pure oils or their nano-formulation counterparts, the RGR and RCR indices decreased, but the FDI of adults increased. The lowest and highest RGR indices, 0.011 and 0.042 mg.mg^{-1}.day^{-1}, were achieved by adults exposed to the LC_{25} and LC_{10} of GEO@CSNPs and CEO@CSNPs, respectively. Moreover, the lowest and the highest RCRs, 0.075 and 0.153 mg.mg^{-1}.day^{-1}, were associated with the LC_{25} of GEO@CSNPs and LC_{10} of CEO@CSNPs, respectively. The lowest (20.85%) and the highest (54.85%) FDI were

Concentration (µl.l^{-1})	Treatment	Repellency mean (%) ± SE	F($_{df}$)	*P*-value
LC_{10}	CEO	23.33 ± 3.33 a	3.57(3,11)	0.06^{ns}
	GEO	36.67 ± 3.33 ab		
	CEO@CSNP	33.33 ± 3.33 ab		
	GEO@CSNP	43.33 ± 6.67 b		
LC_{20}	CEO	36.67 ± 3.33 a	3.61(3,11)	0.06^{ns}
	GEO	43.33 ± 3.33 ab		
	CEO@CSNP	43.33 ± 6.67 ab		
	GEO@CSNP	56.66 ± 3.33 b		
LC_{30}	CEO	46.67 ± 3.33 a	6(3,11)	< 0.05*
	GEO	56.67 ± 3.33 ab		
	CEO@CSNP	56.67 ± 3.33 ab		
	GEO@CSNP	66.66 ± 3.33 b		

Table 4. The repellent effects (mean ± SE) of sub-lethal concentrations of pure CEO and GEO and their respective nano-formulations on *T. castaneum* adults after 24h exposure. In each column for each concentration, means with the same letters are not significantly different using Tukey's test at P-value ≥ 0.05. ns: no substantial difference at *P* value > 0.05. *There is a significant difference (*P*-value < 0.05, Tukey's test).

Concentration (µl. disc^{-1})	Treatment	RGR mean (mg.mg^{-1}. day^{-1}) ± SE	RCR mean (mg.mg^{-1}. day^{-1}) ± SE	FDI mean (%) ± SE	ECI mean (%) ± SE
LC_{10}	Control	0.056 ± 0.003aA	0.019 ± 0.008aA		29.983 ± 1.627aA
	CEO	0.042 ± 0.002 ab	0.153 ± 0.007b	20.853 ± 3.606a	27.747 ± 2.227a
	GEO	0.037 ± 0.003 b	0.122 ± 0.004bc	29.066 ± 2.493a	30.410 ± 3.041a
	CEO@CSNPs	0.037 ± 0.007b	0.135 ± 0.004bc	25.588 ± 2.520a	28.036 ± 5.267a
	GEO@CSNPs	0.032 ± 0.004 b	0.109 ± 0.011c	34.938 ± 6.403a	30.138 ± 4.899a
	Chloroform	0.052 ± 0.003A	0.019 ± 0.007A		29.021 ± 1.819A
LC_{15}	Control	0.052 ± 0.003 aA	0.0181 ± 0.006aA		28.972 ± 2.243aA
	CEO	0.038 ± 0.008ab	0.137 ± 0.005b	27.445 ± 2.351a	27.916 ± 5.665a
	GEO	0.032 ± 0.002b	0.111 ± 0.006c	35.993 ± 4.128ab	29.428 ± 2.207a
	CEO@CSNPs	0.031 ± 0.004b	0.115 ± 0.006bc	35.137 ± 3.772ab	27.979 ± 4.916a
	GEO@CSNPs	0.026 ± 0.001b	0.095 ± 0.007c	43.902 ± 3.880b	27.468 ± 2.172a
	Chloroform	0.050 ± 0.002A	0.018 ± 0.005A		28.370 ± 1.884A
LC_{25}	Control	0.050 ± 0.003aA	0.017 ± 0.007aA		29.868 ± 2.119aA
	CEO	0.034 ± 0.006b	0.118 ± 0.005b	36.807 ± 3.054a	28.567 ± 4.257a
	GEO	0.028 ± 0.002b	0.100 ± 0.003bc	41.715 ± 2.455ab	28.257 ± 2.132a
	CEO@CSNPs	0.029 ± 0.003b	0.098 ± 0.006bc	48.012 ± 3.160ab	30.926 ± 4.213a
	GEO@CSNPs	0.011 ± 0.003c	0.075 ± 0.004c	54.662 ± 1.616b	28.207 ± 0.438a
	Chloroform	0.050 ± 0.002A	0.017 ± 0.008A		29.544 ± 1.746A

Table 5. Nutritional and feeding deterrence indices of *T. castaneum* adults exposed to flour discs treated by sub-lethal concentrations of EOs and their counterpart nano-formulations (data obtained after 72h exposure). Means with the same letters are not significantly different using Tukey's test at P-value ≥ 0.05The LC_{10}, LC_{15}, and LC_{25} obtained from contact toxicity of EOs (based on the active ingredient) against adults *T. castaneum*. The effect of chloroform alone was also compared with the control group using Tukey's test and showed with capital letter. It was not observed any significant effect (P-value ≥ 0.05). In conclusion, it can be applied as solvent for EOs. RGR: Relative growth rate, RCR: Relative consumption rate, FDI: Feeding deterrence index, ECI: Efficacy of conversion of ingested food.

demonstrated by adults exposed to the LC_{10} of CEO and LC_{25} of GEO@CSNPs, respectively. The lowest and the highest ECI of adults were 27.46% and 30.9% related to the LC_{15} and LC_{25} of GEO@CSNPs and CEO@CSNPs, respectively (Table 5).

Nano-formulations, in addition to improving the properties of essential oils and protecting them, affected the repellent activity and nutritional indicators of adults more than pure oils as many studies[29,41,77]. According to our results, EOs@CSNPs significantly affected the repellent activity of *T. castaneum* adults, because CSNPs enhance repellent effectivity and negatively nutritional indices and FDI. The nano-formulations of GEO and CEO promoted their repellent effects on adults at LC_{30} by 15 and 18% compared to pure oil counterparts (Table 3). Furthermore, the repellency of pure oils and nano-formulations were concentration dependent, positively. The higher repellent effect of nano-formulations compared to pure EOs on different species of insects showed in

limited studies[38,39]. The EOs fast evaporation may prevent by using polymer-based nano-formulations, so the repellency was enhanced. Yeguerman et al. found that the repellents effect of peppermint and palmarosa was 12h and 36h, respectively. EOs@NP enhanced their repellent effects over a longer time[48]. In another study, it was observed that two EOs such as *Origanum vulgare* (OV) and *Laurus nobilis* (LN) in PEG-NPs enhanced and prolonged the repellent effect of EOs against *S. oryzae* and *L. serricorne* up to 60 and 48h, respectively[78]. Kumar and Hemalatha showed that repellent activity of PEG-cardamom EO against the storage pest *Oryzaephilus Surinamensis* was concentration dependent, positively[38] that is consistent with our results. In another study, Lima et al. reported that the *Baccharis reticularia* EO repellency against *T. castaneum* adults improved by increasing the concentrations from 8.8 to 17.6 $\mu g.cm^{-2}$ in pure oil and nano-formulations[39].

At low concentrations of nano-formulations such as 1.1 and 4.4 $\mu g.cm^{-2}$, the percentage of repellent activity was 34% and 82%, while in these concentrations, no repellent activity recorded for the pure EOs in contrast to our results. At LC_{30}, the repellent activity of GEO increased by 17% compared to CEO. The same repellency of nano-formulation of GEO was 15% higher than CEO. Although, some components in CEO have good repellent activity on adults, but the identified sulfur thiosulfate compounds, such as diallyl disulfide of GEO was not detected on it. So, GEO has a high percentage of diallyl disulfide as the main volatile and repellent compound against adults, consistent with Plata-Rueda's study[46]. In consistent with our results, González et al. observed the EOs nano-formulation enhances the repellent effects of EOs against german cockroach; NPs increase a higher RI with a longer repellency period than the pure EOs alone. For Bergamot and geranium NPs, repellency enhanced to 72 and 144 h, respectively[29]. The nutritional indicators clarified the EOs effects on the interactions between insects and their food[29].

Decrease RGR and RCR promoted by NPs have a direct relationship with the fecundity and longevity of the adult insect[29]. Generally, based on our results, CSNPs indicated more influence on reducing relative growth and consumption rates compared with pure EOs, especially in high concentrations. Similar effects were indicated by Gonzalez et al. that assessed the effects of EO@NPs against stored product insect pests[40]. Both RGR and RCR were concentration-dependent, negatively. The amount of FDI (percentage) was concentration-dependent, positively. Increasing the concentration of EOs significantly their nano-formulations can reduce the tendency of adults for feeding. The highest amount of FDI observed in adults fed with discs impregnated nano-formulations loaded with GEO and CEO, respectively (Table 4). At LC_{25}, the RGR of *T. castaneum* adults provided on nano-formulations of GEO and CEO decreased by 60% and 14% compared to those provided on their respective pure oils. The nano-formulations of EOs decreased the RCR of adults up to 25% and 11% in GEO and CEO, respectively. The nano-formulations of GEO and CEO increased the FDI of these oils up to 24% and 23%, respectively. Huang et al. indicated that nutritional indices of *S. zeamais* and *T. castaneum* adults were affected by two specific compounds of garlic oil including methyl allyl disulfide and diallyl trisulfide that are mainly feeding and post-digestion inhibition[79]. The same compounds detected in garlic oil but in lower amounts which was reported in our previous study[33].

In addition, the effects of EOs and their nano-formulations on the nutritional parameters of stored pests have been evaluated by various researchers[29,40,80–85]. Our findings were consistent with Gonzalez et al.[40]. They investigated the effect of PEG-geranium and bergamot EOs on the feeding indices of red flour beetle. Nutritional parameters such as RGR, RCR, and ECI changed significantly ($P<0.05$) when nano-formulations applied at 1 and 2 mg. $disc^{-1}$ to the red flour beetle. They didn't observe any significant alternation in nutritional parameters ($P>0.05$) in pure EOs, in contrary with our findings. They confirmed that the different efficacy between pure EOs and their nano-formulations can attribute to the drying of flour discs impregnated with the treatments, so that during 12h many practical components on the nutritional indices evaporated while nano-formulations reduced volatility. Moreover, only at a higher dose (2 mg. $disc^{-1}$), the FDI between nano-formulations and pure EOs was different, significantly. However, in the present study, after 15 min, the flour discs dried, and different concentrations of pure EOs altered nutritional indices, too. Moreover, the RGR and RCR on the studied storage pest for NPs containing EOs decreased more than pure EOs aligned with our results. In another research, the effects of EOs and NPs at LC_{25} and LC_{50} on the nutritional physiology were also evaluated against German cockroach, *Blattella gennanica*. They observed that at LC_{25}, RGR and RCR were significantly reduced by NPs [29].

Yeguerman et al. evaluate the lethal and sub-lethal activity of *Origanum vulgare* (L.) (OV) and *Laurus nobilis* (L.) (LN) EOs loaded in PEG-NPs. They observed the NPs of OV and LN altered the nutritional physiology [78]. In another study, the effect of polymeric nano capsules loaded with *Mentha spicata* and *Mentha pulegium* EOs were investigated on the nutritional indices of *T. castaneum* in different concentrations. The RGR and RCR were concentration-dependent negatively exception in pure *M. spicata* EO [41]. The FDI was concentration-dependent that confirmed our results.

The higher sensitivity of *T. castaneum* adults to nano-formulation compared to pure EOs could be attributed to the enhancement miscibility of prepared EOs in the NPs, uniformity distribution in water followed by more constituents' entrance to the insect's body through their respiratory system[19,86]. Controlled release of EOs from NPs can lead to efficacy over time, while pure EOs are oxidized and evaporated sooner due to higher vapor pressure and direct exposure to the environmental conditions, so their effectiveness reduced. In addition to the physicochemical properties of NPs containing EOs, their small size can improve mobility, distribution, penetration into the peritrophic matrix, and ultimately their different detoxification process in insects[40,41]. The ECI of *T. castaneum* adults treated with GEO was 3% more than CEO, which was not significant. The narrow range of ECI was 25–29% (Table 4) that observed in another studies[41]. ECI is an overall measure of an insect's ability to utilize the food ingested for growth and development[87]. Therefore, no change in ECI values indicates that ingested secondary biochemical do not exhibit any chronic toxicity[88]. The highest ECI values in the pure garlic oil indicated a higher conversion efficiency of ingested and digested food to body biomass. The effects of ECI conversion efficiency felt when the tested insect was in the larval stage of its life with ravenous appetite and

wide range of diets. At the same time, in the present study, the adults tested did not feed a lot from flour discs, so it does not affect some indicators such as ECI.

Conclusion

In this study, GEO and CEO encapsulated in CSNPs in order to improve the physicochemical properties and enhance the lethal and sub-lethat effectivity of *T.castaneum* in environmental condition. For this purpose, CSNPs containing EOs prepared using the ionic cross-linking method. Then, the sub-lethal toxicity of GEO@CSNPs and CEO@CSNPs against storage pests compared with pure EOs. Basd on the repellency and nutritional indices, formulated oils in nano structure could increase the sub-lethal effectivity of EOs loaded in CSNPs. Moreover, applying nanoformulations in concentrations equivalent to oils promoted their sub-lethal effectivity for an extended period. Ultimately, the impact of GEO@CSNPs and CEO@CSNPs on the lethal and sub-lethat effect on *T.castaneum* showed that these nanoformulations are appropriate materials in integrated pest management programs. It should be noted that due to the high volume of biometric analysis, the tests should perform at different times and on different days at the same hours, observing uniform environmental conditions. However, insects were a mixture of male and female and it was not clear how many males and females there are, feeding experiments showed many differences. They were repeated several times to obtain uniform results. Additional in vivo and in vitro bioassays such as sub-lethal efficient of CSNPs loaded with various oils against different developmental stages of *T. castaneum* and other insects can be performe and results compare to each other.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Author contributions

GhM was the supervisor of the project. GhM and FKh designed the research. NF conducted experiments for NP preparation. HA contributed GC analysis. FKh analyzed data and GhM, NF and HA approved results. FKh wrote the manuscript draft and GhM and NF revised it. All authors read and approved the manuscript.

Competing interests

The authors declare no competing interests.

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