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Toxicity, Chemical Composition and Molecular Docking of Sweet Orange and Lemon Cypress Essential Oils Tested against *Tribolium castaneum* (Coleoptera: Tenebrionidae)

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Abstract

The red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) is one of the most destructive pests attacking stored grains all over the world. Related problems with using synthetic chemical insecticides to control such insect, have strongly promoted the need for safe alternatives such as plant essential oils (EOs). This study aimed to assess the contact and fumigant toxicity of two EOs extracted from sweet orange fruit peels and lemon cypress leaves against the adult stage of *T. castaneum*. Both EOs were found to be effective against *T. castaneum* adult. Probit analysis revealed that, sweet orange EO was more effective than lemon cypress in contact and fumigation bioassays. Gas Chromatography and Mass Spectrometry analysis (GC-MS) showed that, sweet orange oil mainly consisted of D-limonene (97.38%) while, sabinene (19.14%) and 3-Cyclohexen-1-ol,4-methyl-1-(1-methylethyl)-(cas) (12.17%) were the major constituents of lemon cypress EO. The expected interaction between the main compounds of the two tested oils and acetyl choline esterase (AChE) enzyme of *T. castaneum* were proved by computational docking programs. Major identified compounds showed varying levels of binding affinities to AChE, where; (R)-1-isopropyl-4-methylcyclohex-3-en-1-ol (BE= -6.27 kcal/ mol) and sabinene (BE= -5.63 kcal/ mol) in lemon cypress EO had the highest and lowest binding affinity, respectively. This study suggested that these major compounds combined with each other in both oils could be responsible for the insecticidal and AChE inhibitory potentials of both EOs.

Keywords *Tribolium castaneum* , Sweet orange , Lemon cypress , Essential oils, GC-MS, Molecular docking

1. Introduction

Sufficient production of food for an increasing human population has been an issue of a global concern. It is estimated that the global population size will increase by 46% in 2050, which will require increasing of agricultural production to guarantee food security [1]. Production of food and insurance of its security are the challenges the world face where insect and disease damage and climate change are major constraints [2]. The stored grains are greatly important to many nations [3], and its infestation by insect pests represents a great risk to grains and their products [4]. Hundreds of hexapods and other arthropods infest stored food commodities, about 600 species among which belong to order Coleoptera [5]. Insect pests of stored grain are responsible for 10-40% of annual lose worldwide [6]. Post-harvest losses due to stored-product insects are estimated to be 9% in developed countries and 20% or more in developing countries [7]. *T. castaneum* is considered the main pest of stored grains [8]. Fumigation with synthetic chemicals has been proved to be one of the most economic ways of controlling insect pests of stored products [9]. However, the control of such pests using fumigants of synthetic pesticides had some limitations such as; environmental disturbances, pest resurgence and resistance to pesticides, lethal effects on non-target species, increasing cost of application and poisoning of farm workers and consumers [10].

The growing awareness of these risks led to the search for safe new methods to control such pests [11]. Products based on plant extracts, phyto-oils and purified substances of plant origin can be an alternative to the conventional pesticides [12]. Citrus, the genus of the family Rutaceae [13] has fruits which are important sources of EOs with wide application [14].

EO derived from orange peels was recognized to possess toxic, poor development and feeding deterrent effects on *Rhyzopertha domonica*, *Sitophilus oryzae* and *T. castaneum* [15]. Lemon cypress as a medicinal plant belongs to family Cupressaceae and contains large amounts of monoterpenes, sesquiterpenes or diterpenes in its EOs of fresh and dried leaves. Lemon cypress is a plant traditionally used by the population as insect repellent and treatment of many diseases including amoebiasis and malaria [16]. The traditional usage of this plant is attributed to its ability as a reservoir of active molecules against malaria parasites and disease vectors [17]. The aim of the present study is to evaluate the insecticidal activity of two EOs extracted from the fruit peels of sweet orange, (*Citrus sinensis L. Osbeck*) Rutaceae and the leaves of lemon cypress,

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(*Cupressus macrocarpa* cv. Goldcrest) *Cupressaceae* against *T. castaneum* adult focusing on their chemical composition. It also aimed to perform a molecular docking analysis to recognize the energy of binding affinity (Kcal/mol), the amino acids interactions and the binding sites of the major constituents of both EOs against the active site of AChE.

2. Materials and methods

2.1. Insect rearing

T. castaneum adults reared for several generations (more than ten generations) were collected and successfully reared on the Egyptian organic wheat grains (*Triticum aestivum* var Sakha 95) according to [18].

2.2. Tested native plants

Fruit peels of sweet orange and leaves of lemon cypress were collected from the private botanical garden, Faculty of Agriculture, Benha University, Qalyubia, Egypt. Specimens of sweet orange and lemon cypress were carefully cleaned, dried in the shade and ground into powder. The ground samples were packed in plastic bags and stored in the refrigerator at 4°C until further extraction.

2.3. Extraction of essential oils

Powders of the ground plant samples were subjected to hydro-distillation in a Clevenger–type apparatus for three hours [19]. The extracted essential oils were separated and dried using anhydrous Na_2SO_4. EO samples were kept in dark-brown glass vials and stored in a refrigerator adjusted at 4°C till further GC-MS analysis and biological evaluations.

2.4. Bioassay tests

Bioassay tests were performed through both contact and fumigation methods. Wheat grains were frozen at -18°C for two weeks before application of EOs to eliminate any possible infestation by any pest. All experiments were conducted using sexed 1-2 weeks-old *T. castaneum.*

2.4.1. Contact bioassay

Efficiency of two EOs as contact toxicants was conducted against *T. castaneum* according to [20]. Stock oils solutions were diluted in petroleum ether, and seven concentrations (4, 6, 8, 10, 12 and 16 %) v/v for sweet orange EO and (16, 18, 20, 22, 24, 26 and 28 %) v/v for lemon cypress EO were prepared. Each concentration was mixed and shaken with cracked wheat grains (1 ml /10 gm wheat) for ten minutes in a sterile jar and then left to dry. All concentrations were replicated three times. Twenty adults of *T. castaneum* insects were separately added to each jar and then the glass jars were covered with muslin and then kept under controlled conditions of (28 ± 2°C and 65 ± 5% RH). The same procedure was used with petroleum ether only as a control. Mortality of *T. castaneum* adults was calculated after 3 and 5 days and corrected according to Abbott's formula [21]. Probit analysis was applied to determine toxicity values (LCs) for each tested oil and the slope of its regression line, [22].

2.4.2. Fumigation bioassay

Application of tested EOs by fumigation was conducted as a modification of the method mentioned by [23] a number of wooden boxes, each of dimensions; (20 x 20 x 20 cm), which represented the fumigant chambers (figure 1A), were prepared. Number of small mosquito electric repellent devices (ERD; e.g, RAID*) were purchased, fixed in the middle of the box (1ERD/box) and connected to electricity from outside source. Mats (7 cm^2 area) originally prepared for use in the ERD (Figure 1 B) were purchased and used after removing the repellent material on the mat. Eliminating the repellent material on the mat was done when the ERD carrying a mat was operated for 48 hours.Change the color of mat from green to white proved the elimination of the mosquito repelling material which followed by rinsing in petroleum ether and drying with hot air to ensure the absence of any smell A large number of the mats were cleaned and kept for use in the following experiments. 28 wooden boxes and 28 ERDs were prepared before starting the planned tests. Figure 1 (A, B) illustrates the picture of the designated "fumigation chamber" with its accessories. To cover the box from the upper side, a glass slab was used.

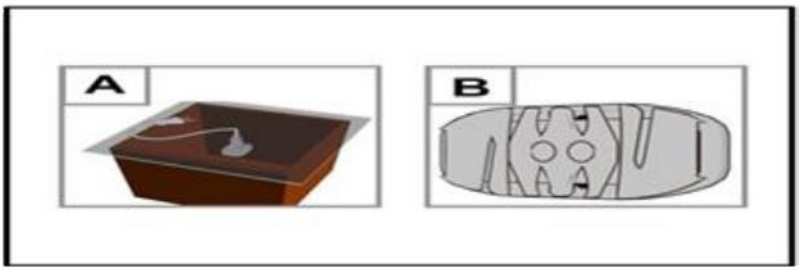

Fig. 1: Schematic diagram of designed fumigation chamber with it's accessories (A) A wooden box with electric repellent device (ERD) containing the mat and covered with a glass slab (The complete fumigation test chamber), (B) A mat inserted in the electric repellent device (ERD).

2.4.3. Test procedure

Based on the preliminary trials to study the fumigant toxicity of both EOs, stock solutions were prepared using petroleum ether. A series of concentrations ranging between (30-80 & 60-90 %) v/v were tested in case of sweet orange and lemon cypress EOs, respectively. Preparation of the tested concentrations was performed by impregnating the clean mat in 0.7 ml of each concentration. Then, the solvent on mats was allowed to evaporate for 1 hour at room temperature. Six concentrations of both EOs with three replicates were prepared. Each replicate was provided with 20 insect of *T. castaneum* adult. Another box provided with ERD carrying mat, free from any oil, dried from solvent treatments and containing the same number of insects was used as control treatments. Boxes were covered and the ERDs were connected to the electric power source and kept at

room temperature for 20 and 30 hours exposure periods. Mortality was computed and adjusted based on [21], Probit analysis [22] was applied to determine toxicity values (LC) values for each tested oil and the slope of its regression line.

2.5. Chemical analysis of essential oils

Components of sweet orange and lemon cypress EO samples were analyzed and identified via GC-MS tool under the same previous conditions [24-25]. The chemical constituents were identified depending on (i) AMDIS software (Automated Mass spectral Deconvolution and Identification), (ii) spectral collection of the Wiley Library, (iii) The library of NIST database (Gaithersburg, MD, USA; Wiley, Hoboken, NJ, USA).

2.6. Molecular docking

Molecular docking is a useful tool for understanding the ligand- receptor interaction that may help in the prediction of the ligand mechanism of action. To investigate the interaction between the major compounds of two EOs and AChE, a molecular docking study was carried out using the auto-dock vina version 1.1.2. The crystal protein was downloaded from Uniprot (Uniprot code: AF-A0A139WIV4). Preparation of the targeted protein was done by removing water molecules, correcting unfilled valence atoms, and minimizing the energy of protein peptides by applying CHARMM force fields. Additionally, the tested compounds were drawn using Chem-Bio Draw Ultra17.0 and saving them in SDF file format. The tested ligands were protonated, and energy was minimized using MMFF94 force field and the minimized structures were saved for molecular docking. Molecular docking was performed by docking algorithms using blind docking technique, where the target enzyme protein was held rigid, and the ligands were allowed to be flexible. Each molecule was allowed to produce twenty different interaction poses with the protein during the refinement. The docking scores (affinity free energy) of the best-fitted poses with the active site of the *T. castaneum* AChE were recorded. Two and three dimensional binding modes (2D & 3D) were generated using Discovery Studio 2016 visualizer software.

2.7. Statistical analysis

Dosage mortality data were estimated by Probit analysis [22] using a computer program [26]. Other obtained data were analyzed as one/two way ANOVA, using Proc ANOVA in SAS statistical software package [27]. Means were calculated in the same operation and compared by Duncan multiple range tests at 0.05 probability level (P = 0.05) [28].

3. Results

3.1. Insecticidal efficiency of essential oils

The obtained data in table 1 illustrated the contact toxicity of sweet orange and lemon cypress EOs tested against *T. castaneum* adults for 3 & 5 days where percentage mortality was found directly proportional with the concentrations of two oils and exposure time. A significant difference was noticed by comparing the mortality values for both EOs (at all tested concentrations) with the control group at three and five days post- exposure (table 1 $P = 0.0001$). There were no significant differences between mortality readings of *T. castaneum* after three and five days post application of sweet orange EO ($P > 0.05$). Unlike sweet orange EO, lemon cypress EO achieved a significant difference in mortality values between three and five days post exposure

Table 1: Percentage mortality (mean ± SE) of *T. castaneum* adults exposed for 3 and 5 days to various concentrations of wheat grains treated with sweet orange and lemon cypress EOs following contact bioassay

Plant essential oils	Conc. % (v/v)	Accumulative mortality % after different periods (days)	
		3	5
Sweet orange	4	20 ± 1.7 f	23.3 ± 2.5 g
	6	38.3 ± 0.96 e	41.7 ± 0.96 f
	8	53.3 ± 0.96 d	55 ± 1.7 e
	10	65.0 ± 1.7 c	66.7 ± 1.7 d
	12	75.0 ± 2.9 b	76.7 ± 2.5 c
	14	81.7 ± 0.96 ab	83.3 ± 1.7 b
	16	88.3 ± 1.7 a	90 ± 0.96 a
	Control	0.0 ± 0.00 g	0.0 ± 0.00 h
		$F_{7,16}$=175.13	$F_{7,16}$=200.94
		P = 0.0001	P = 0.0001
Lemon cypress	16	18.3 ± 3.3 g	23.3 ± 1.9 g
	18	30 ± 2.9 f	35 ± 1.7 f
	20	41.7 ± 2.9 e	46.7 ± 0.96 e
	22	51.7 ± 2.9 d	56.7 ± 0.96 d
	24	61.7 ± 2.9 c	66.7 ± 1.7 c
	26	70 ± 2.9 b	76.7 ± 0.96 b
	28	78.3 ± 3.3 a	85 ± 1.7 a
	Control	0.0 ± 0.00 h	0.0 ± 0.00 h
		$F_{7,16}$=147.58	$F_{7,16}$=117.11
		P = 0.0001	P = 0.0001

Different letters within columns indicate significant difference at $P \leq 0.05$

Based on LC_{50} values in table 2, sweet orange EO (7.4 % & 7%) was more effective than lemon cypress EO (21.6 % & 20.5%) at three and five days post treatment.

Results in table 3 showed the effect of fumigant application of two EOs against *T. castaneum* adult after 20 & 30 hours. The gradual increase in concentrations of oils and exposure period noticeably led to an increase in *T. castaneum* adult mortality.

Although the fumigant toxicity caused by two oils were not high, a significant difference in the mean mortality was achieved between adult insects treated with two tested EOs (at all tested concentrations) and those in the control group at 20 &30 hours of exposure (*table 3 P* = 0.0001). Overall, exposure of *T. castaneum* adults for sweet orange EO recorded a significant difference between mortality values at 20 & 30 hours post treatment. Otherwise, mortality means didn't significantly differ at 20 & 30 hours of treatment with lemon cypress EO.

Table 2: Lethal concentrations values of sweet orange and lemon cypress EOs recorded against *T. castaneum* adults at 3 & 5 days as time variants following contact bioassay

Plant essential oil	Exposure periods (days)	LC_{25} (v/v) (95% fiducial limits)	LC_{50} (v/v) (95% fiducial limits)	LC_{95} (v/v) (95% fiducial limits)	Slope (±SE)	Degrees of freedom
Sweet orange	3	4.6 (3.7-5.8)	7.4 (5.9-9.2)	23.3 (18.6-29.1)	3.3±0.05	5
	5	4.3 (3.5-5.5)	7 (5.6-8.8)	22.5 (17.9-28.3)	3.2±0.05	5
Lemon cypress	3	17.2 (15.5-19.1)	21.6 (19.5-24)	37.7 (34-41.9)	6.8±0.02	5
	5	16.5 (14.9-18.2)	20.5 (18.5-22.7)	35 (31.6-38.7)	7.1±0.02	5

LC = the lethal concentration for 25%, 50% and 95% of treated insects (SE) Standard Error

Table 3: Percentage mortality (mean ± SE) of *T. castaneum* adults exposed for 20 & 30 hours to various concentrations of wheat grains treated with sweet orange and lemon cypress EOs following fumigation bioassay

Plant essential oil	Conc. % (v/v)	Accumulative mortality % after different periods (hours)	
		20	**30**
Sweet orange	**30**	15 ± 2.9d	18.3 ± 1.7 e
	40	25 ± 2.9d	30 ± 2.9 d
	50	36.7 ± 4.4c	41.7 ± 1.7 c
	60	45 ± 2.9bc	50 ± 5 bc
	70	53.3 ± 1.7ab	56.7 ± 4.4 ab
	80	60.0 ± 5.8a	63.3 ± 4.6 a
	Control	0.0 ± 0.00e	0.0 ± 0.00 f
		$F_{6,14}$=40.18	$F_{6,14}$=45.56
		P = 0.0001	P = 0.0001
Lemon cypress	**60**	18.3 ± 1.7 e	18.3 ±1.7 e
	65	25 ± 0 de	26.7 ± 2.9 d
	70	30 ± 2.9 cd	31.7 ± 1.7 c
	75	36.7 ± 3.3bc	36.7 ± 3.3 bc
	80	41.7 ± 1.7 b	43.3 ± 3.3 ab
	90	51.7 ± 4.4 a	53.3 ± 3.3 a
	Control	0.0 ± 0.00 f	0.0±0.00 f
		$F_{6,14}$=44.75	$F_{6,14}$=45.56
		P = 0.0001	P = 0.0001

Different letters within columns indicate significant difference at $P \leq 0.05$

Reading of LC_{50} values displayed in table 4 showed that , sweet orange EO (65.8% & 60.5%) was more efficient as a fumigant toxicant against *T. castaneum* adult compared to lemon cypress EO (87.7 % & 86.3%) at 20 & 30 hours post exposure.

Table 4: **Lethal concentrations values of sweet orange and lemon cypress EOs recorded against *T. castaneum* adults at 20 & 30 hours of exposure following fumigation bioassay**

Plant essential oil	Exposure periods (hours)	LC_{25} (v/v) (95% fiducial limits)	LC_{50} (v/v) (95% fiducial limits)	LC_{95} (v/v) (95% fiducial limits)	Slope (±SE)	Degrees of freedom
Sweet orange	**20**	39.5 (30.7-50.8)	65.8 (51.1-84.6)	228.3 (177.5-293.7)	3.04± 0.05	4
	30	35.5 (27.4-46.1)	60.5 (46.7-78.6)	222.6 (171.5-288.7)	2.9±0. 05	4
Lemon cypress	**20**	65.5 (56.8-75.7)	87.7 (75.9-101.3)	178.4 (154.5-206.1)	5.3±0. 03	4
	30	64.8 (56.3-74.6)	86.3 (75-99.3)	173.3 (150.5-199.4)	5.4±0. 03	4

LC = the lethal concentration for 25%, 50% and 95% of treated insects (SE) Standard Error

3. 2. Chemical analysis of essential oils

The obtained data from GC-MS chemical analysis of sweet orange EO as shown in table 5 revealed the presence of five bioactive compounds, which accounted for 100% of the total oil composition. Monoterpene compounds represented the major chemical class which constituted the main components of oil structure (100%). The major monoterpene compound was; D-Limonene (97.38%). The other compounds were found in minor percentages as following; á-Myrcene (1.51%), cis-Ocimene (0.61%), Sabinene (0.36%) and 3-Carene (0.14%). Analysis of lemon cypress EO displayed in table 6 using GC chromatogram totally demonstrated forty two compounds representing 99.97% of the total oil structure. The characterized compounds were mainly categorized in five chemical classes including; monoterpenes which involved; monoterpenes hydrocarbons (53.31%) and oxygenated monoterpenes (38.87%). Fatty acids as the second group occupied (3.28%) of total oil composition. Sesquiterpenes compounds comprised the third chemical class which maintained; sesquiterpenes hydrocarbons (2.93%) and one oxygenated sesquiterpenes compound (0.34%). While the fourth class was; diterpenes compounds which included diterpenes hydrocarbons (0.54%) and one oxygenated diterpenes compound (0.27%).

Table 5: Chemical components of EO obtained from sweet orange peels, *C. sinensis*

S/N	Compound name	RT	Area (%)	Chemical class
1	D-Limonene	5.55	97.38	MH
2	à-Myrcene	4.72	1.51	MH
3	cis-Ocimene	3.79	0.61	MH
4	Sabinene	4.47	0.36	MH
5	3-Carene	5.10	0.14	MH
Monoterpenes			**(100%)**	
hydrocarbons			**(100%)**	
Total identified				

(RT) - Retention time, (MH) –Monoterpenes Hydrocarbons

Phenylpropanoid was the last and the least class represented only in (0.43%) from the total structure of the oil. Sabinene (19.14%) and 3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-(cas) (12.17%) were the major compounds.

Table 6: Chemical components of EO obtained from lemon cypress leaves, *C. macrocarpa* by GC-MS

S/N	Compound name	RT	Area (%)	Chemical class
1	à-Thujene	6.47	5.41	MH
2	à-Pinene	6.64	3.35	MH
3	Camphebe	6.93	1.36	MH
4	Sabinene	7.50	19.14	MH
5	2-à-Pinene	7.57	0.16	MH
6	à-Myrcene	7.79	4.21	MH
7	l-Phellandrene	8.13	0.18	MH
8	à-Terpinene	8.43	5.64	MH
9	dl-Limonene	8.73	4.59	MH
10	ç-Terpinene	9.42	6.43	MH
11	à-Tepinolene	10.13	2.84	MH
12	Trans sabinene hydrate	9.51	0.67	OM
13	Tricyclo[4.4.0.0(3,8)]dec-9-en-4-ol	10.0	0.17	OM
14	1,6-Octadien-3-ol, 3,7-dimethyl-	10.25	2.32	OM
15	Thujone	10.59	0.16	OM
16	2-Cyclohexen-1-ol,1-methyl-4- (1- methylethyl)-, cis	10.86	1.24	OM
17	2-Bornanone(+) -	11.23	6.35	OM
18	Citronella	11.44	3.74	OM
19	Isopulego2	11.72	0.24	OM
20	1-Borneol	11.92	0.19	OM
21	3-Cyclohexen-1-ol,4-methyl-1- methylethyl)-(cas)(	12.32	12.17	OM
22	3- Cyclohexene-1-methanol, à,à,4-trimethyl-,(S)-(CAS)-	12.50	1.49	OM
23	Terpinene-3-ol	12.68	0.25	OM
24	à-Citronellol	13.47	9.72	OM
25	Citronellyl acetate	16.35	0.16	OM
26	Citronellic acid	15.60	0.85	FA
27	Geranyl butyrate	28.20	0.22	FA
28	4-Fluoro-2-acetylphenol	12.91	0.45	FA
29	2-Undecanone	14.95	0.25	FA
30	2-Tridecanone	19.50	1.14	FA
31	2-Hydroxy-2,4,4-trimethyl-3-(3-methylbuta-1,3-dienyl)cyclohexanone	21.94	0.20	FA
32	Cyclopentane carboxylicacid,3-methylene-,1,7,7-trimethylbicyclo [2.2.2]hept-2-yl ester	27.46	0.17	FA
33	Caryophyllene	18.30	0.63	SH
34	à-Humulene	19.30	0.24	SH
35	Thujopsene -12	19.40	1.02	SH
36	Bicyclogermacrene	19.87	0.23	SH
37	Spiro[5.5]undec-2-ene,3,7,7-trimethyl-11-methylene-, (-)-	20.02	0.81	SH
38	Nerolidol	20.96	0.34	OS
39	Kaur-15-ene,(5à,9à,10á)-	28.61	0.37	DH
40	7-Isopropyl-1,1,4atrimethyl 1,2,3,4,4a,9,10,10a-octahydrophenanthrene	29.68	0.17	DH
41	Sclareol	30.94	0.27	OD
42	Methyl eugenol	17.18	0.43	PH
Monoterpenes hydrocarbons			(53.31%)	
Oxygenated monoterpenes			(38.87%)	
Fatty acids			(3.28%)	
Sesquiterpenes hydrocarbons			(2.93%)	
Oxygenated sesquiterpenes			(0.34%)	
Diterpenes hydrocarbons			(0.54%)	
Oxygenated diterpenes			(0.27%)	
Phenylpropanoids			(0.43%)	
Total identified			(99.97%)	

*(*RT) - retention time , (MH) –Monoterpenes Hydrocarbons , (OM) – Oxygenated Monoterpenes , (FA) – Fatty Acids, (SH) – Sesquiterpenes Hydrocarbons, (OS) – Oxygenated Sesquiterpenes, (DH)–Diterpenes Hydrocarbons, (OD) – Oxygenated Diterpenes and (PH) – Phenylpropanoids

3.3. Molecular docking study of essential oils active compounds with acetylcholine-esterase of *T. castaneum*.

A computational approach was carried out to focus on the occurred binding between *T. castaneum* protein involved in neurological responses i.e acetylcholine- esterase and the dominant compounds of the two EOs and to interpret the insecticidal activity of both oils. D-Limonene and á-Myrcene; the main identified compounds in EOs of sweet orange (*C. sinensis*) and Sabinene and (R)-1-isopropyl-4-methylcyclohex-3-en-1-ol from lemon cypress (*C. macrocarpa*); were docked separately into the active pocket of AChE to investigate the nature of binding with the crucial enzyme and discover some insights about the mode of action.

Results of binding of these compounds with AChE were presented in table 7 and figure 2--D**)**. D-limonene; the major identified compound in sweet orange EO showed a good binding affinity to *T. castaneum* AChE with binding energy of; -5.74 kcal/mol. Although D-limonene did not form any hydrogen bonds (H-bonds), it formed six hydrophobic π-Alkyl interactions with His248, Tyr480, Tyr122, and Arg488 (figure 2A). Additionally, à-myrcene showed acceptable binding affinity to *T. castaneum* AChE with affinity score of-5.64 kcal/mol.

Table 7: The binding data for the major compounds identified in sweet orange and lemon cypress EOs with ACHE target site of *T. castaneum*

S/N	Compound name	BE (ΔG) (Kcal/mol)	RMSD value (Å)	Interactions	
				Hbs	π - interactions
1	D-Limonene	-5.74	1.14	0	6
2	à-myrcene	-5.64	1.24	0	4
3	Sabinene	-5.63	0.77	0	5
4	(R)-1-isopropyl-4-methylcyclohex-3-en-1-ol	-6.27	0.67	2	3

BE = Binding energy (Kcal/mol), RMSD = Root-mean-square deviation, Hb_s =Hydrogen bonds

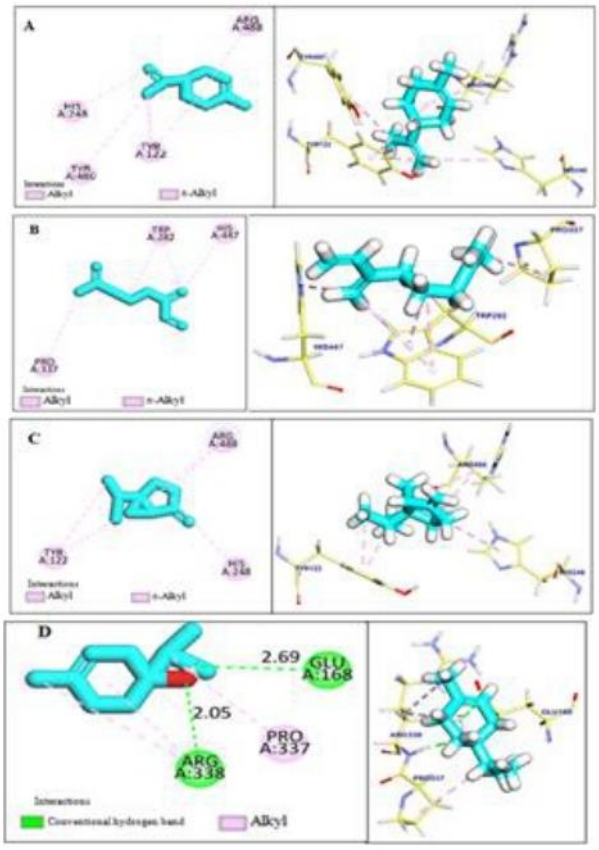

Fig. 2: Two and three dimensional binding modes (2D & 3D) of major identified compounds in sweet orange and lemon cypress EOs, respectively docked in active pocket of *T. castaneum* AChE (A) D-limonene, (B) à-myrcene , (C) sabinene, and (D) (R)-1-isopropyl-4-methylcyclohex-3-en-1-ol, respectively.

Similarly, à-myrcene did not form any H-bonds and formed four hydrophobic π-Alkyl interactions with Pro 337, Trp 282, and His 447 (figure 2B). Moreover, interaction of the major identified compound from lemon cypress EO; sabinene (BE= -5.63 kcal/ mol) showed acceptable binding affinity to *T. castaneum* AChE without forming any H-bonds. Sabinene created also five hydrophobic π-Alkyl interactions with Tyr122, His248, and Arg488 (figure 2 C). (R)-1-isopropyl-4-methylcyclohex-3-en-1-ol (BE= -6.27 kcal/ mol) from lemon cypress EO showed a strong binding affinity to *T. castaneum* AChE by creating three hydrophobic π-Alkyl interactions with Arg338, and Pro337. In addition to its hydrophobic contacts with the amino acids, it was the only compound forming H-bonds with two active site residues; Arg 338, and Glu168 with distances of 2.05 Å, and 2.69 Å (figure 2D). Major identified compounds of two EOs based on molecular docking results including binding energy could be arranged according to their binding affinity and strength of binding mode with *T. castaneum* AChE as following; (R)-1-isopropyl-4-methylcyclohex-3-en-1-ol with the lowest binding energy in lemon cypress oil > D-limonene, > à-myrcene > sabinene which had the highest binding energy in lemon cypress oil. Results displayed in Figure 3 revealed that, D-limonene (sweet orange EO) and sabinene (lemon cypress EO) occupied the same binding position, while, à-myrcene (sweet orange EO) and (R)-1-isopropyl-4-methylcyclohex-3-en-1-ol (lemon cypress EO) demonstrated the same binding site. The similarity of binding sites between D-limonene and sabinene can be observed from the interaction with the same amino acid residues (His248, Tyr122, and Arg488). On the other hand, à-myrcene and (R)-1-isopropyl-4-methylcyclohex-3-en-1-ol showed the same binding interaction with Pro337.

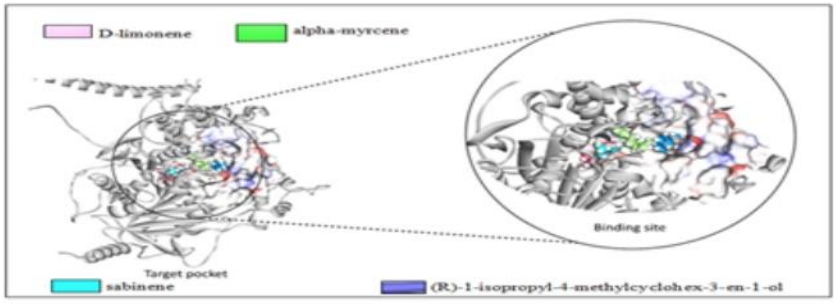

Fig. 3: Binding sites of D-Limonene (pink), à-myrcene (green), Sabinene (torquate) and (R)-1-isopropyl-4-methylcyclohex-3-en-1-ol (blue) occupying the target site of AChE of *T. Castaneum*

4. Discussion

Botanical pesticides including plant EOs are eco-friendly and effective substitutes to synthetic chemical insecticides [29]. EOs synthesized by aromatic plants play an important role in protecting plants against insect pests. These compounds affect insects via insecticidal, deterrent, repellent and antifeedant activities [10]. Essential oils were recognized as contact insecticides [30-31] and volatiles acting like fumigants offering the potential for protection of stored-product [32-33]. The present study proved the contact and fumigant toxicity of two EOs extracted from sweet orange and lemon cypress plants growing in Egypt against *T. castaneum* insect. Data in table 1 and 3 revealed a linear relationship between insect mortality with both of oils' concentration and exposure periods. Similar finding was proved by [34] after treatment of *T. castaneum* with turmeric EO. Results shown in table 2

proved the high insecticidal activity of sweet orange EO in contact application. In agreement with the obtained results, [35] proved high potency of EO obtained from sweet orange peels against *T. castaneum* in contact application. Similar finding was also reported by [36] who tested *C. sinensis* EO against *T. castaneum* adults after 168 hours. Lemon cypress EO also, exerted high contact toxicity against *T. castaneum* adult as maintained intable 2. In harmony with study's findings, *C. macrocarpa* EO extracted from the Egyptian leaves showed toxicity effect on *Synthesiomyia nudiseta* after 24 hours [37]. Similarly, *C. macrocarpa* EO showed insecticidal activity against *Culex pipiens* adults [38]. Comparing LC_{50} values of both EOs in contact application indicated higher potency of sweet orange EO than lemon cypress. Similar results were recorded by [39]. Higher efficiency of sweet orange EO against *T. castaneum* adult compared to lemon cypress could be attributed to the higher content of D-limonene as documented by [40].

Concerning fumigant application, sweet orange EO was found to be effective fumigant toxicant against *T. castaneum*. This finding was confirmed for *C. sinensis* EO tested for 24 and 48 hours against adults of *T. confusum* [41] and *T. castaneum* [42]. Lemon cypress EO demonstrated moderate fumigant toxicity against *T. castaneum* as shown in table 4. Saad and Abou-Taleb, [43] found the same mild fumigant toxicity action after treatment of *Spodoptera littoralis* 4th larval instar with *C. macrocarpa* EO. Mild fumigant toxicity effect was exerted against *Theba pisana* adults after application of *C. macrocarpa* EO [44]. Moderate fumigant toxicity of lemon cypress EO vapor could back to the quite weak inhibition of acetyl cholinesterase activity caused by *C. macrocarpa* oil [45]. The mode of action by which the tested EOs affected *T. castaneum* insect could participate in clarification the toxicity of these oils. Insect mortality due to EOs was caused through their monoterpenoids components [46] which acted as neurotoxins, with many suggested modes of action including; inhibition of acetyl cholinesterase, antagonism of GABA and agonism of octopamine [47]. In addition to the ease penetration of cell

membranes declining membrane transport and potential, ion equilibrium and quick intervene in the physiological functions of insects, mitochondrial dysfunction which may lead to cell death [48]. Based on LC_{50} values for sweet orange and lemon cypress EOs, contact application was more effective in protection of stored wheat grains by inducing higher mortality between *T. castaneum* adults than fumigation. But no one can ignore the role of fumigation application as a method to manage stored product pests by killing the concerned pests with avoidance further damage to the infested commodities [49].

Volatile components of sweet orange EO, *C. sinensis*, in the present results, were commonly consistent with the findings of [50] and [51]. But, the slight difference in chemical composition and percentages of constituents in sweet orange EO could be due to the ecological zone, climate, time of harvesting, genetic results, vegetative stage, and extraction processes [52]. Chemical composition of lemon cypress EO, *C. macrocarpa* proved the dominance of sabinene which was confirmed by [53]. While [54] found that, Terpinen-4-ol was the major compound in the Egyptian *C. macrocarpa* EO leaves followed by Sabinene and β-Citronellol. The difference could back to several factors, such as origin, nutritional conditions, pesticide use, latitude, drying and harvest time [55].The toxicity of EO is related to its chemical composition [56]. The present study highlighted that, the monoterpenoid D-limonene as major component in sweet orange EO was supposed to induce it's toxicity against *T. castaneum*, a finding confirmed by [15] and [57]. The effectiveness of lemon cypress EO could also be explained by its chemical components. Sabinene as the major component of the oil could contribute to it's toxicity. According to the present study, contact application of lemon cypress EO was preferable compared to the moderate fumigant action which could be related to weak fumigant toxicity of sabinene [58]. Sabinene appeared to be highly toxic against larvae of *C. quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti* [59].

Results of molecular docking study revealed that, (R)-1-isopropyl-4-methylcyclohex-3-en-1-ol compound in lemon cypress EO had the greatest potential binding affinity and the best binding mode with the active site of *T. castaneum* AChE making it the most probable compound inhibiting AChE activity. The best binding mode of (R)-1-isopropyl-4-methylcyclohex-3-en-1-ol compound was correlated to the lowest binding energy where, such compound had a better stability between the ligand and the receptor and thus the bond formed supposed to be strong [60]. Furthermore, the best binding profile of this compound was due to the interactions with the amino acids at binding site of *T. castaneum* AChE represented in hydrophobic interactions and H-bonds formed due to the chemical structure of such compound containing hydroxyl group. It was previously mentioned that, the chemical structure of linallol, menthone and carvone compounds of *Mentha* EOs including hydroxyl group played a vital role in binding to AChE of *Reticulitermes dabieshanensis* through forming different alkyl interactions and H- bonds with AChE [61]. Unfortunately, activity of (R)-1-isopropyl-4-methylcyclohex-3-en-1-ol as expectable inhibitor of AChE enzyme due to the presence of hydroxyl group wouldn't seem to induce the toxicity of lemon cypress EO against *T. castaneum* insect. Polarity of hydroxyl group [62] increases the hydrophilicity of such compound making it dissimilar to the polar waxy cuticle of insect and thus fails to penetrate insect cuticle. Otherwise, ability of alkaloid and phenolic compounds to form complexes with insect cuticle lipid layers helped them to penetrate inside the insect body, reach to the nervous system and inhibit AChE activity [63].

D-limonene in sweet orange EO could be also, a probable inhibitor of AChE through forming the highest number of alkyl interactions with AChE. D-limonene was proved to inhibit AChE activity in *S. ceamais* [64] and *Haemonchus contortus* [65]. Based on the molecular docking results, it was shown that both of D-limonene and Sabinene had the same binding position with the amino acid residues. So, we could conclude that sabinene probably had the potential like D-limonene to act as ACHE inhibitor. Surprisingly, limonene and sabinene had fumigant insecticidal activities as AChE inhibitors against *S. oryzae* [66]. Collectively, the insecticidal activity of sweet orange and lemon cypress EOs against *T. castaneum* adult beetle could be relied on the binding appearing between D-Limonene and à-myrcene compounds (sweet orange EO) and Sabinene and R)- 1-isopropyl-4-methylcyclohex-3-en-1-ol compounds (lemon cypress EO) with AChE in molecular docking profiles .

But, it was unlikely that this research could confirm the speculation that the activity of both oils was due to these compounds only. Because, the present study only focused on the major compounds as probable inhibitors of *T. castaneum* AChE. In addition, other compounds even were represented with small amount in these oils; they probably had similar inhibitory effects against AChE, D-limonene (97.38%) was the most dominant component in sweet orange EO which was probably responsible for it's toxicity while, Sabinene (19.14%) as the major constituent in lemon cypress EO furnishing the activity. Therefore, this requires more study of all compounds to understand how they affect the insect.

5- Conclusion

From the current study, it can be concluded that sweet orange and lemon cypress EOs had great potential effect against *T. castaneum* and could be used as tools in its control. Contact application was found to be effective than fumigation. GC-MS analysis indicated that, D-limonene, dominant component in sweet orange EO was more effective than Sabinene as the major constituent in lemon cypress EO. Based on the molecular docking results, both D-limonene and Sabinene had the same binding position with the amino acid residues. Sabinene probably had the potential like D-limonene to act as AChE inhibitor. According to the current data, tested essential oils may be effective approach to protect wheat grains from *T. castaneum* infestation as they contain a range of bioactive compounds which are selective and have little or no harmful effect on the environment and the non-target organisms including humans. In conclusion, these oils showed insecticidal activity and exhibited great promise in suppressing populations of *T. castaneum*.

6. Conflict of interest

There are no conflicts to declare.

7. Declarations

Ethics approval and consent to participate no human or vertebrate animals were included in this study. Ethical approval for the study was obtained from Ain Shams University, Faculty of Science, Research Ethics Committee (REC) (approval code ASU-SCI/ ENTO/ 2025/1/6)

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