SUPPLEMENTARY MATERIAL	1
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Insecticidal properties of essential oils against <i>Tribolium castaneum</i>	3
(Herbst) and their inhibitory effects on acetylcholinesterase and	4
adenosine triphosphatases	5
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Abstract	13
Essential oils from twenty Egyptian plants were obtained by hydrodistillation. The	14
chemical composition of the isolated oils was identified by gas chromatograph/mass	15
spectrometer (GC/MS). Fumigant and contact toxicities of the essential oils were	16
evaluated against the adults of <i>Tribolium castaneum</i> . In fumigation assays, the oil of	17
<i>Origanum vulgare</i> (LC ₅₀ = 9.97 mg/l air) displayed the highest toxicity to adults of <i>T.</i>	18
<i>castaneum</i> . In contact assays, the oils of <i>Artemisia monosperma</i> (LC ₅₀ = 0.07 mg/cm ²)	19
and <i>O. vulgare</i> (LC ₅₀ = 0.07 mg/cm ²) were the most potent toxicants to the adults of	20
<i>T. castaneum</i> . Biochemical studies showed that the tested oils caused pronounced	21
inhibition of acetylcholinesterase (AChE) and adenosine triphosphatases (ATPases)	22
isolated from the larvae of <i>T. castaneum</i> . The oil <i>C. macrocarpa</i> (IC ₅₀ = 12.3 mg/l)	23
was the most potent inhibitor to AChE, while the oil of <i>C. viminalis</i> (IC ₅₀ = 4.4 mg/l)	24
was the most potent inhibitor to ATPases.	25
Keywords: Essential oils; Fumigant toxicity; Contact toxicity; <i>Tribolium castaneum</i> ;	26
Acetylcholinesterase; Adenosine triphosphatases	27
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Experimental	35
Insects	36
A colony of rust red flour beetle, <i>Tribolium castaneum</i> (Herbst) (Coleoptera: Tenebrionidae), was maintained in our laboratory for over 12 years without exposure to insecticides. The insect was reared on wheat flour mixed with yeast (10:1, w/w). Insect rearing was carried out at 26±1°C and 65±5% RH in complete darkness. The unsexed adult insects used in toxicity studies were 2-week post-emergence. Larvae used for the biochemical studies were 15 days old.	37 38 39 40 41 42
Plant materials	43
Twenty plant species including <i>Artemisia judaica</i> L. (aerial parts), <i>Artemisia monosperma</i> Del. (leaves), <i>Astoma seselifolium</i> DC. (aerial parts), <i>Callistemon viminalis</i> (Sol.ex Gaertn.) G. Don (leaves), <i>Citrus aurantifolia</i> (Christm.) Swingle (fruit peels), <i>Citrus lemon</i> (L.) Burm.f. (fruit peels), <i>Citrus paradisi</i> Macfad (fruit peels), <i>Citrus sinensis</i> (L.) Osbeck (fruit peels), <i>Cupressus macrocarpa</i> Hartw. ex Gordon (leaves), <i>Cupressus sempervirens</i> L. (leaves), <i>Myrtus communis</i> L. (leaves), <i>Origanum vulgare</i> L. (aerial parts), <i>Pelargonium graveolens</i> L'Her (leaves), <i>Pituranthos tortuosus</i> (Desf.) Benth (aerial parts), <i>Rosmarinus officinalis</i> L. (leaves), <i>Syzygium cumini</i> L. Skeels (leaves), <i>Schinus molle</i> L. (leaves), <i>Schinus terebinthifolius</i> Raddi (leaves), <i>Thuja occidentalis</i> L. (leaves) and <i>Vitex agnus-castus</i> L. (leaves) were collected during the flowering stages from different locations of Alexandria, Albehira and Matrouh Governorates, Egypt, in August, 2011 to April, 2012. The plant materials were identified by Prof. FathAllah Zaitoon of Plant Pathology Department, Faculty of Agriculture, Alexandria University. Voucher specimens have been deposited in Department of Chemistry of Pesticides, Faculty of Agriculture, Alexandria University.	44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59
Isolation of essential oils	60
Essential oils were extracted by hydrodistillation in a Clevenger-type apparatus for 3 h. The leaves and aerial parts were partially dried at room temperature (26±1°C) with good ventilation for five days and the fruit peels were used fresh. Anhydrous sodium sulfate was used to remove water after the extraction. Extracted oils were stored in airtight glassware in a refrigerator at 4°C.	61 62 63 64 65
Analysis of essential oils	66
Chemical analyses were performed on a gas chromatography (Hewlwett Packard 5890)/mass spectrometry (Hewlwett Packard 5989B) (GC-MS) apparatus. Essential	67 68

oils were diluted in diethyl ether and a sample amount of 0.5 μ l was injected. The GC column was a 30 m (0.25 mm i.d., film thickness 0.25 μ m) HP-5MS (5% diphenyl) dimethylpolysiloxane capillary column. The GC conditions were as follows: injector temperature, 240°C; column temperature, isothermal at 70°C and held for 2 min, then programmed to 280°C at 6°C/min and held at this temperature for 2 min; ion source temperature, 200°C; detector temperature, 300°C. Helium was used as the carrier gas at the rate of 1 ml/min. The effluent of the GC column was introduced directly into the ion source of the MS. Spectra were obtained in the EI mode with 70 eV ionization energy. The sector mass analyzer was set to scan from 40 to 400 amu for 5 s. The oil components were identified by comparison of their retention indices and mass spectra with the NIST Mass Spectral Library.

Fumigant toxicity assay

The toxicity of essential oils vapors was tested against *T. castaneum* adults by using a modified fumigant toxicity assay as described by Huang et al. (2000). Glass jars (1 l) were used as fumigation chambers. Essential oils were applied to Whatman No.1 filter paper pieces (2×3 cm) attached to the undersurface of the screw caps of jars at: 2.5, 5, 7.5, 10, 15, 20, 30, 40, 50, 80, 90, and 100 mg. The inner side of the jar's neck was painted with Vaseline to prevent direct contact of insects with monoterpenes. Caps were screwed tightly onto the jars containing 20 adults of *T. castaneum* and the lid was sealed with parafilm. Control insects were kept under the same conditions without essential oils. Four replicates of each treatment were set up. All treated and untreated insects were maintained at 26±1°C, 65±5% RH and a 12:12 h light:dark photoperiod. The number of dead insects was recorded after 24 h of treatment. Insects were considered dead when no leg or antennal movements were recorded. The lethal concentration causing 50% mortality (LC₅₀) expressed as mg/l air were calculated from log-concentration mortality regression lines (Finney 1971).

Contact toxicity assay

The insecticidal activity of twenty essential oils against the adults of *T. castaneum* was evaluated by direct contact application assay (Qi and Burkholder, 1981; Broussalis et al., 1999). A series of concentrations was prepared in acetone. One milliliter of each concentration was applied on the bottom of a glass Petri dish (9 cm diameter) to give a range of concentrations (0.01–1.0 mg/cm²). Solvent was allowed to evaporate for 2 min prior to introduction of insects. Twenty adults were placed into each Petri dish. Control dishes were treated with acetone alone. All treatments were

replicated four times. Malathion was used as a reference insecticide. Mortality percentages were recorded after 24 h of treatment, and LC₅₀ values were calculated.

In vitro inhibition of acetylcholinesterase (AChE) activity assay

Larvae of *T. castaneum* (6 g) were homogenized in 20 ml of 50 mM ice-cold phosphate buffer (pH 7.4) using a Polytron Kinemetica. Homogenates were filtered through two layers of cheesecloth. The filtrates were centrifuged under cooling (5,000 rpm for 30 min at 4°C), and supernatants were used as the enzyme source. Inhibition of AChE was determined by the colorimetric method of Ellman et al. (1961) using ATChI as substrate. Enzyme aliquots (50 µl) and dithiodinitrobenzoic (DTNB) (100 µl of 0.01 M) were added to 0.1 M phosphate buffer (pH 8.0; 2.8 ml). To this mixture, essential oil solutions (20 µl) prepared in acetone and Triton-X 100 (at concentration of 0.01%) were added. The essential oils were tested at a series of concentrations (5, 10, 15, 20, 30, 50, 75, 100, 150, 250 and 500 mg/l). Control treatments were prepared by adding 20 µl of acetone without essential oil. Mixtures were incubated at 37°C for 15 min. Reactions were started by adding ATChI (30 µl) followed by incubation at 37°C for 10 min. The change in absorption at 412 nm was monitored on Sequoia-Turner Model 340 spectrophotometer. All the experiments were done in triplicate. Specific activity of AChE (ΔOD/mg protein/min) was calculated for each concentration and control. Inhibition percentage of AChE activity was calculated as follows: AChE inhibition % = $[1 - SA_T / SA_C] \times 100$, where SA_T is specific activity of the enzyme in treatment and SA_C is specific activity of the enzyme in control. The concentrations of the tested essential oil that inhibited the hydrolysis of substrate by 50% (IC₅₀) were determined by a linear regression analysis (probit analysis) between the inhibition percentages against the oil concentrations.

In vitro inhibition of total adenosine triphosphatases (ATPases) activity assay

Larvae of *T. castaneum* were homogenized in an appropriate volume (10% w/v) of TSE buffer (40 mM Tris-HCl, 320 mM sucrose, 1mM EDTA, pH 7.4). The homogenates were centrifuged at 5,000 rpm for 30 min at 4°C using IEC-CRU 5000 cooling centrifuge. The supernatants were recentrifuged at 15,000 rpm using Cryofuge 20-3, Heraeus Christ centrifuge for 15 min at 4°C. The particulate pellets were resuspended in the homogenizing TSE buffer solution to give approximately 1-2 mg protein/ml. The *in vitro* inhibition of total ATPases activity was determined by incubating the enzyme for 30 min at 37°C with different concentrations (1, 2, 4, 8, 10,

15, 20, 40, 80 and 100 mg/l) of essential oils prepared in acetone. Triton-X 100 was added to enzyme solution at concentration of 0.01%. Then, total ATPases activity was determined as reported by Koch et al. (1969) with slight modification in which Tris-HCl buffer was used instead of imidazole buffer. Total volume of reaction mixture was 850 μ l contained 40 mM Tris-HCl pH 7.4, 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 5 mM ATP and the enzyme source (10 μ l), which contains the appropriate protein concentration. The mixture was incubated at 37°C for 15 min, and then reaction was terminated by addition of 150 μ l of TCA (30 % w/v). Librated inorganic phosphorus (Pi) from ATP hydrolysis was measured according to Taussky and Shorr (1953) by addition of 4 ml coloring reagent (10 % Amm. Molybdate in 10 N H_2SO_4 ; diluted to 1:10 by GDW, then $FeSO_4$ was added to the resultant diluted solution). The color was measured at 740 nm against blank using Sequoia-Turner Model 340 spectrophotometer. The concentration of Pi was calculated from a standard curve and accordingly the enzyme specific activity was computed as μ mole Pi /mg protein/min. The standard curve of Pi was made using KH_2PO_4 (concentrations from 0.1-1.0 μ mole/ml). To 1 ml of each concentration, 4 ml of coloring reagent mentioned above were added. The developed color was measured at 740 nm. Results were plotted and a standard curve was fitted using the least squares method ($K = 0.8$). Inhibition percentage of ATPases activity and IC_{50} of the tested oils were calculated as previously described.

Statistical analysis

The mortality of each concentration was calculated after 24h of treatment as the mean of four replicates. The mortality and enzyme inhibition percentages were subjected to probit analysis (Finney 1971) to obtain the LC_{50} and IC_{50} values, using SPSS 12.0 (SPSS, Chicago, IL, USA). The values of LC_{50} and IC_{50} were considered significantly different if the 95% confidence limits did not overlap.

Acknowledgments

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Table Caption:	208
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Table S1. Major constituents of the essential oils isolated from Egyptian plants	210
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Table S1

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Plant oil	Compound (%)	Plant oil	Compound (%)
<i>Artemisia judaica</i>	β -Thujone (49.83) Chrysanthenone (10.88) α -Thujone (8.21)	<i>Myrtus communis</i>	α -Pinene (26.16) 1,8-Cineole (16.45) Linalool (11.23)
<i>Artemisia monosperma</i>	Capillene (36.86) Capillin (14.68) γ -Terpinene (12.46)	<i>Origanum vulgare</i>	Pulegone (77.45) Menthone (4.86) <i>cis</i> -Isopulegone (2.22)
<i>Astoma seselifolium</i>	Sabinene (23.02) 4-Terpineol (17.83) γ -Terpinene (8.97)	<i>Pelargonium graveolens</i>	β -Citronellol (35.92) Geraniol (11.66) Citronellylformate (11.40)
<i>Calistemon viminalis</i>	1,8-Cineole (71.77) α -Pinene (11.47) Terpinen-4-ol (3.18)	<i>Pituranthos tortuosus</i>	Sabinene (32.09) 4-Terpineol (20.31) Myristicine (6.84)
<i>Citrus aurantifolia</i>	Limonene (40.19) β -Pinene (19.65) α -Citral (8.14)	<i>Rosmarinus officinalis</i>	1,8-Cineole (19.60) Camphor (17.01) α -Pinene (15.12)
<i>Citrus lemon</i>	Limonene (56.30) β -Pinene (8.81) γ -Terpinene (6.42)	<i>Syzygium cumini</i>	α -Pinene (17.26) α -Terpineol (13.88) β -Pinene (11.28)
<i>Citrus paradisi</i>	Limonene (74.29) Linalool (4.61) Linalool oxide (4.18)	<i>Schinus molle</i>	α -Phellandrene (29.87) β -Phellandrene (21.08) Elemol (13.00)
<i>Citrus sinensis</i>	Limonene (89.23) Linalool (2.98)	<i>Schinus terebinthifolius</i>	Sabinene (14.93) γ -Elemene (13.18) β -Elemene (6.63)
<i>Cupressus macrocarpa</i>	Terpinen-4-ol (20.29) Sabinene (18.67) β -Citronellol (13.01)	<i>Thuja occidentalis</i>	α -Pinene (35.49) δ -3-Carene (25.42) α -Cedrol (9.05)
<i>Cupressus sempervirens</i>	α -Pinene (37.88) δ -Carene (20.05) α -Terpinolene (6.91)	<i>Vitex agnus-castus</i>	<i>trans</i> -Caryophyllene (15.19) 1,8-Cineole (13.04) Bicyclogermacrene (7.30)

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