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Acaricidal and Antioxidant Activities of Anise Oil (*Pimpinella anisum*) and the Oil's Effect on Protease and Acetylcholinesterase in the Two-Spotted Spider Mite (*Tetranychus urticae* Koch)

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Abstract: The two-spotted spider mite, *Tetranychus urticae*, also known as the red spider, is one of the most harmful pests in agriculture and causes large losses of many crops. These mites have rapidly developed a resistance to many chemical pesticides in recent years. In this study, the essential oil of seeds of the anise plant (*Pimpinella anisum*) was extracted by hydrodistillation, and the chemical composition of the oil was analyzed. The antioxidant activity of the volatile oil was determined by the DPPH radical scavenging assay. The acaricidal activity of the anise oil, a natural acaricide, was evaluated for its ability to protect green bean plants from mite injury. The two-spotted spiders were spread on green bean seedlings for 1 week; then, different plants were sprayed with different concentrations of anise oil (10, 20, 30, or 40 µL/L). Our results revealed that anethole was the major component of anise oil, at 53.23%. The acaricidal effect of the various concentrations on *T. urticae* was recorded after 24, 48, and 72 h of treatment. Our findings suggest that anise oil showed significant acaricidal activity against *T. urticae* in a dose- and time-dependent manner. Anise oil at a concentration of 40 µL/L killed 96.0% of the red spiders after 72 h. Also, all concentrations of anise oil inhibited acetylcholinesterase, and the spiders' protease activity declined when the plants were treated with 30 or 40 µL/L of anise oil. The concentrations of 10 and 20 µL/L did not significantly affect the protease activity of *T. urticae* mites. We can conclude that anise oil exhibited acaricidal activity against *T. urticae* and that this was highly correlated with the inhibition of acetylcholinesterase and protease activities in the mites.

Keywords: *Tetranychus urticae*; acaricidal activity; *Pimpinella anisum*; anise oil; AChE; protease

1. Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch (Acari family Tetranychidae), is a harmful pest that damages many crops, including vegetables, fruits, and ornamental plants [1]. It is also the most common plant-eater of the family Tetranychidae [2], attacking 3877 host crops of both greenhouse and field crops [1]. The two-spotted spider mite is polyphagous; it can feed on hundreds of plants, including many that are important for the economic well-being of countries. In controlling this pest, the main option is the use of synthetic acaricides, but they are not always efficient, because this species has a high ability to develop resistant populations [3], and because these acaricides are not selective for predatory mites [4]. The improper use of these agents can result in environmental and food contamination, especially of fruits and vegetables meant to be consumed when freshly harvested [5]. Chemical acaricides such as organophosphorus compounds, synthetic pyrethroids, and amitraz are used to control mites. Recently, many acaricides have been replaced with newer, safer agents owing to the toxicity resistance of and environmental damage caused by earlier agents [6]. Several essential oils were evaluated for their acaricidal activities. Essential oils consisting of volatile secondary metabolites, mostly terpenes [7], act as botanical insecticides [8,9], some of which are marketed as commercial ingredients for pesticides [10]. Many essential oils are used as insecticides due to their direct effects, biodegradability, and low level of toxicity to mammals [10–12]. Also, these natural compounds have very low toxicity in humans, making them good alternatives to synthetic acaricides. These essential oils, including anise oil, contain many natural compounds that have acaricidal activity [8,9]. The insecticidal and acaricidal activities of anise oil could be attributed to their terpenoidal content [7]. As previous studies have shown, the toxicity of anise oil against insect pests is due to its active ingredients, such as (e)-isoeugenol, limonene, linalool, and α-pinene [13,14]. Amini et al. [14] identified the components of essential oils and showed that *Pimpinella anisum* L. had the most fumigant toxicity against the storage pests. Anise insecticidal activity was also studied by Tunç and Sahinkaya [15], who verified some anise acaricide activity against *Tetranychus cinnabarinus* Boisd. On fumigation tests, Lucca et al. [16] reported that an important potential insecticidal characteristic of anise was that it repelled moths. Anethole phenylpropanoid, the most important component of anise, was very efficient in controlling *Aedes aegypti* and *Culex pipiens* L. mosquitoes [17]. The volatile essences (monoterpenes) attract insects, mainly sucking insects, due to their nutrimental attributes [18]. Koul et al. [19] reported that the efficiency of essential oils such as anise is variable and depends on the presentation, application, and concentration of the metabolite used. In the attempt to identify alternatives with less environmental impact for the control of the two-spotted spider mite, this study aimed at evaluating the acaricidal effect of anise oil on females of this species, including the maximal lethal concentrations of the oil. It also investigated the relationship between the acaricidal activity of anise oil and its inhibitory effect on spider protease and acetylcholinesterase (AChE) activities.

2. Materials and Methods

2.1. Materials

The seeds of the anise plant (*P. anisum* L.) were purchased from the local market in Cairo, Egypt. The substances 1,1-diphenyl-2-picrylhydrazyl (DPPH); butylated hydroxyanisole (BHA); 5,5-dithiobis (2-nitrobenzoic acid) (DTNB); acetylthiocholine iodide (ATCh); and bovine serum albumin (BSA) were purchased from the Sigma–Aldrich Chemical Co (Taufkirchen, Germany). All reagents used throughout this study were of analytical grade.

2.2. Extraction of Essential Oil of Anise

The essential oils of dried anise seeds (200 g) were isolated by steam distillation for 3 h using a glass Clevenger-type apparatus. The extracted yellow-colored volatile oils were

dried over anhydrous Na_2SO_4 and were kept at 4 °C in dark glass vials for further analysis.

2.3. Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

After evaporation, the extracted oil residue was dissolved with 3 mL ethyl acetate and 1 mL transferred to GC vial for GC/MS analysis. Gas chromatography-mass spectrometry was used for the analysis of various components of anise volatile oil that were present in modest quantities, in addition to the analysis of the main components of anise essential oil. The identification of components was based on a comparison of their mass spectra and retention time with those of the authentic compounds and by computer matching with the NIST and WILEY libraries as well as by comparison of the fragmentation pattern of the mass spectral data with those reported in the literature. The analysis was carried out using a GC (Agilent Technologies 7890A, Poway, CA) interfaced with a mass-selective detector (MSD, Agilent 7000, Poway, CA) equipped with a polar Agilent HP-5 ms (5%-phenyl methyl poly siloxane) capillary column (30 m × 0.25 mm i.d. (In diameter) and 0.25 μm film thickness). The carrier gas was helium with a linear velocity of 1 mL/min. The injector and detector temperatures were 200 °C and 250 °C, respectively. Volume injected was 1 μL of the sample. The MS operating parameters were as follows: ionization potential 70 eV, interface temperature 250 °C, and acquisition mass range 50–800 [20].

2.4. DPPH Free Radical Scavenging Activity

The capacity of essential anise oil to scavenge the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical was determined according to the method described in [21]. Butylated hydroxyanisole (BHA) was used as a reference. In the DPPH method, 500 μL of freshly prepared of DPPH solution (50 μM in absolute ethanol) was mixed with 300 μL of anise oil (10–50 μL/L) and left in the dark for 30 min. Then, the absorbance of the mixture was recorded at 517 nm. The capability to scavenge the DPPH radical (% inhibition) was calculated using the following equation: % inhibition = $[(A_c - A_t)/A_c] \times 100$. Where A_c is the absorbance of the reaction without sample (control) and A_t is the absorbance of test samples.

2.5. Mite Rearing

One adult female mite was transferred by a fine camel hair brush to a sweet leaf disc (1 mm, in diameter), preserved on a humid cotton wool pad in a Petri dish, and left for 24–48 h to allow it to lay eggs. The deposited eggs were preserved under laboratory conditions at 27 ± 2 °C, 60 ± 5 % R.H. (Realtive Humidity) and 16 L: 8 D photoperiod until hatching. The newly hatched larvae were transferred singly to fresh sweet potato leaves to follow their development. Distilled water containing 0.01% Tween 80 was used to prepare the five dilutions of anise oil as an emulsifier and to make it slightly sticky. Distilled water containing 0.01% Tween 80 was used as a control [22].

2.6. Acaricidal Activity of Anise Oil against Tetranychus Urticae under Laboratory Conditions

Four concentrations of the essential oil were used in the experiment (10, 20, 30, and 40 μL/L), and an untreated control group was prepared under laboratory conditions of 27 ± 2 °C and 60 ± 5% relative humidity to evaluate the eggs and adult females of the two-spotted spider mite. The concentrations of the essential oil were chosen based on previous studies and the effectiveness of similar plants on other spiders. Thirty adult females and 120 eggs of *T. urticae* were chosen and used per three replicates. Each group was transferred to a leaf disc of 4 cm^2 in area, which was placed upside down on moist cotton wool in a Petri dish. Each leaf disc had 5 female mites and 20 mite eggs on it. Two mL of each concentration of the anise oil was sprayed onto the surface of the leaf discs using a hand-held atomizer at a distance of 25 to 30 cm. The replicates of the eggs and

adult females and the control were sprayed with distilled water containing 0.01% Tween 80. After 24, 48, and 72 h, the numbers of live and dead adult females were counted. The numbers of hatching eggs also were counted over 6 days by a dissecting microscope. The percentage of mites that had died was calculated and corrected according to Abbott's formula [23].

The values of LC_{50} and LC_{90} were determined using the mortality regression lines drawn according to the Finney, 1952 [24] method and the Sigmaplot program version 2.0 software [25].

2.7. Determination of Enzyme Activities

Adult females of the two-spotted spider mite were homogenized at 4 °C in a 0.066 M, pH 7.5 sodium phosphate buffer with 0.2 percent (*v*/*v*) of Triton X-100. The mixture was centrifuged at 10,000× *g* at 4 °C for 15 min. The protease and acetylcholinesterase activity were measured in supernatants.

2.8. Assay of Protease Activity

The activity of proteases in the whole mite was measured using the Ortego et al. [26] method. A working solution of bovine serum albumin (BSA) was made by diluting the standard BSA solution of 1 mg/mL to 0.1 mg/mL. In 5 test tubes 200 µL, 400 µL, 600 µL, 800 µL, and 1 mL of the BSA working solution was taken and named 1–5. By adding distilled water, the volume was kept at 1 mL. Only distilled water was used to produce a control. 500 µL of the crude extract was collected by Bradford method, and 500 µL of sterile distilled water was added to obtain a final volume of 1 mL. The Bradford reagent was added in 5 mL increments, and the absorbance was measured spectrophotometrically at 595 nm after 5 min of incubation. To assess the proteolytic activity of the crude extract, 50 µL of BAS (1 mg/mL) standard solution and roughly 20 µL of crude extract were used. The BSA and enzyme were mixed together, and the volume was kept at 200 µL by adding phosphate buffer. After 30 min, 2.3 mL of Bradford reagent was added to the mixture. After 5 min, the absorbance was measured against a blank using a spectrophotometer at 595 nm, with two controls: enzyme only and BSA only. The activity of the protease was determined using the Bradford equation [27]. At pH 9.0 at 60 °C, one unit of protease activity is defined as the amount of enzyme that produces 1 µmol of tyrosine per minute.

2.9. Assay of Acetylcholinesterase (AChE) Activity

Acetylcholinesterase was determined according to Wu and Miyata [28] with ATCh iodide as a substrate in the presence of 5,5-dithiobis (2-nitrobenzoic acid) DTNB in a 0.066 M phosphate buffer, pH 7.8, at 25 °C. Absorption was measured at 412 nm. The reaction mixture (2.0 mL) consisted of 0.6 mM ATCh, 0.4 mM DTNB and 0.05 mL aliquot of the enzyme solution.

2.10. Molecular Docking of E-Anethole in AChE

The molecular docking was performed using AutoDock 4 [29]. The system was prepared in PyMOL (Schrödinger), using the plug-in developed by Daniel Seeliger (https://github.com/ADplugin/ADplugin, accessed on 15 August 2021). Acetylcholinesterase and Cathepsin L were obtained from the structure of the AChE (6XYU) [30] and Cathepsin L (3F75) [31] complexes. A 54 × 60 × 54 Å grid box with 29.488 × 71.531 × 12.27 grid point spacing of 0.375° A in the case of Acetylcholine esterase and a 56 × 54 × 40 Å grid box with 24.97 × 18.609 × 35.567 grid point spacing of 0.375° A in the case of Cathepsin L were employed. The ligands structure was drawn using the PubChem draw structure tool (https://pubchem.ncbi.nlm.nih.gov/#draw=true, accessed on 15 August 2021). The default parameter set of autodock4 was used to generate 10 docking poses. The pose with the best energy score was selected as the most representative

2.11. Statistical Analysis

The collected data were calculated as means and standard deviations from three replicates and were analyzed using SPSS statistical software (IBM SPSS Statistics, version 23, New York, NY, USA). The differences between treatments were compared using one-way analysis of variance (ANOVA) according to method of Tamhane and Methods, 1977 [32], post hoc LSD test was also performed at $p \leq 0.000$.

3. Results

The results of the chemical composition study of anise seeds are shown in Table 1. The components of anise oil were analyzed using gas chromatography–mass spectrometry (GC-MS) to determine the active constituents of anise seed oil. The results were as follows: There are a total of 28 components found in anise oil (see Table 1). The major constituent of anise oil is *trans*-anethole (53.23%), followed by estragole (13.52%) and longifolene (6.08%).

Table 1. Chemical composition (%) in essential oil of Pimpinella anisum analyzed by GC-MS.

No Peak	RT [1] (Min)	Component Names	% [2]
1	5.215	α-Pinene	0.59
2	4.621	*E*-β-Ocimene	0.93
3	5.802	D-Limonene	1.00
4	6.171	γ-Terpinene	0.60
5	6.421	3-Carene	0.93
6	6.901	Linalool	0.77
7	6.958	α-Santalol	0.70
8	7.545	Estragole	13.52
9	8.147	*Z*-Anethole	1.17
10	8.734	*E*-anethole	53.23
11	9.099	α-Guaiene	0.88
12	9.238	2-Allyl-4-methylphenol	0.59
13	9.372	(−)-Aristolene	1.47
14	9.501	Caryophyllene	1.26
15	9.718	Aromandendrene	0.56
16	9.803	γ-Elemene	1.07
17	9.976	α-Himachalene	1.94
18	10.218	Longifolene	6.08
19	10.358	Thujopsene	1.26
20	10.489	Cedrene	0.80
21	11.031	Ledene	1.42
22	11.354	Isospathulenol	0.71
23	11.826	*E*-Sesquisabinene hydrate	0.66
24	12.683	*E*-Isoeugenol	4.81
25	13.048	Phenol, 2-methoxy-4-(1-propenyl)-	0.66
26	13.676	Acetophenone, 2′,5′-dimethoxy-	0.71
27	17.561	Geranyl isovalerate	0.99
28	20.336	Heptacosane	0.72

[1] Retention time; [2] Compound percentage.

The minor constituents in the essential oil of anise seed were *cis* anethole, D-limonene, (−)-aristolene, caryophyllene, γ-elemene, α-himachalene, longifolene, thujopsene and ledene. While seventeen components were present at less than 1%. These were: α-pinene (0.59%), T-β-ocimene (0.93%), γ-terpinene (0.6%), 3-carene (0.93%), lin-

alool (0.77%), α-santalol (0.7%), α-guaiene (0.88%), 2-allyl-4-methylphenol (0.59%), aromandendrene (0.56%), cedrene (0.8 %), isospathulenol (0.71%), *trans*-sesquisabinene hydrate (0.66%) *trans*-isoeugenol (4.81 %), phenol, 2-methoxy-4-(1-propenyl)-(0.66%), acetophenone, 2′,5′-dimethoxy-(0.71%), geranyl isovalerate (0.99%) and heptacosane (0.72%).

There are usually considerable variations in the major active compounds within this species. The major component of anise oil seen in the current study was *trans*-anethole, which was similar to major component of many regions of the world. In current research, the main compound of anise oil is *trans*-anethole followed by estragol.

To evaluate the antioxidant activity of anise oil, the free radical scavenging activity against DPPH was determined. The DPPH assay (Figure 1) showed that the antioxidant activity of anise oil at 10 and 20 µL/mL was approximately 36.02 ± 2.033% and 43.62 ± 1.071%, respectively, compared with BHA used as a reference. The antiradical activity percentages of anise oil at 30, 40, and 50 µL/mL were 58.12 ± 1.238%, 68.42 ± 2.007%, and 77.58 ± 1.044%, respectively. The highest percentage of radical scavenging activity (77.58%) was recorded for *P. anisum* at a concentration of 50 µL/mL, followed by a concentration of 40 µL/mL (68.42%). It was noticed that as the concentration of any anise extract increased, the antioxidant activity also increased, possibly due to the increase in the concentration of the active substances in the oil.

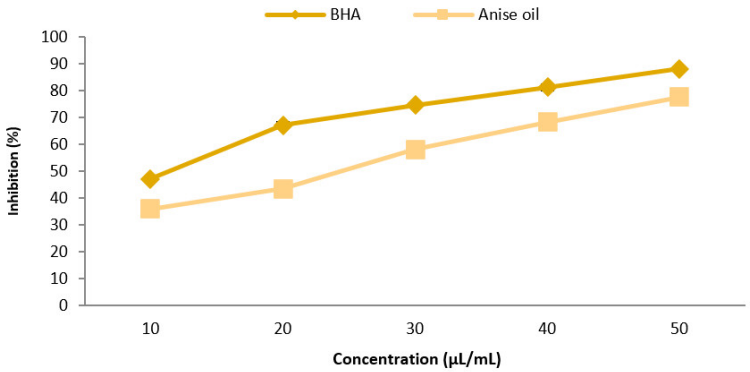


Figure 1. Free radical scavenging activity of essential anise oil (expressed as % inhibition) compared to Butylated hydroxyanisole (BHA).

Table 2 shows the acaricidal activity of various concentrations of anise oil. The obtained data indicated that five concentrations of anise oil showed remarkable acaricidal effects after 24, 48, and 72 h of treatment. Anise oil treatments lead to 91.0 and 96.0% of adult females of *T. urticae* killed after 48 and 72 h of treatment with concentrations of 40 µL/L. The lowest percentages recorded were 27.30%, 28.10%, and 33.00%, respectively, at 10 µL/L after 24, 48, and 72 h. Also, the data clearly indicated that the acaricidal activity of anise oil was increased by increasing both the concentration of the oil and the length of time of the treatment; there were significant differences in the mean values of the tested concentrations on mortality percentages.

Table 2. The mortality percentage of *T. urticae* as affected by different concentrations of anise oil.

Anise Oil Concentration (µL/L)	Mortality% of *T. urticae*		
	24 h	**48 h**	**72 h**
10	27.3 ± 2.7 ^{d}	28.1 ± 1.0 ^{d}	33.0 ± 1.4 ^{d}
20	42.2 ± 3.9 ^{c}	43.9 ± 1.3 ^{c}	45.3 ± 1.8 ^{c}
30	70.1 ± 1.8 ^{b}	73.9 ± 4.1 ^{b}	87.0 ± 1.5 ^{b}
40	89.3 ± 6.6 ^{a}	91.0 ± 1.4 ^{a}	96.0 ± 2.2 ^{a}
Control	0.000	0.000	0.000
L.S.D	0.46	0.98	0.36

Each value is average of three replicated samples ± SD. Different letters refer to significant differences at $p \leq 0.000$.

Results (Table 3) revealed that the mean numbers of eggs hatched was not recorded on the first and second days after treatment for all concentrations of oil. Highly significant differences were recorded on the fourth, fifth, and sixth days. Egg hatchability was observed on the third, fourth, fifth, and sixth days for all concentrations. The lowest mean numbers of eggs hatched were recorded at high concentrations of the oil; the findings for 40 µL/L on the fifth and sixth days were 4.75 ± 0.28 and 1.25 ± 0.28 eggs. The highest values were observed on the third and fourth days at a concentration of 10 µL/L and were 19.75 ± 0.40 and 19.5 ± 0.48 eggs, respectively.

Table 3. Mean numbers of eggs hatched into larvae of *T. urticae* as affected by different concentrations of anise oil.

Conc. (µL/L)	Mean Number of Egg Hatchability ± SE after Detected Days					
	1st	2nd	3rd	4th	5th	6th
10	0.00	0.00	19.75 ± 0.40 ^{a}	19.52 ± 0.48 ^{a}	17.25 ± 0.37 ^{a,b}	11.25 ± 0.31 ^{b}
20	0.00	0.00	19.25 ± 0.28 ^{a}	16.75 ± 0.38 ^{b}	15.25 ± 0.40 ^{b}	9.50 ± 0.29 ^{b}
30	0.00	0.00	16.25 ± 0.39 ^{b}	10.75 ± 0.35 ^{c}	9.50 ± 0.29 ^{c}	6.25 ± 0.20 ^{c}
40	0.00	0.00	10.75 ± 0.21 ^{c}	7.75 ± 0.28 ^{d}	4.75 ± 0.28 ^{d}	1.25 ± 0.28 ^{d}
Cont.	0.00	0.00	19.12 ± 1.00 ^{a}			
L.S.D			1.17	1.52	3.33	1.79

Each value is average of three replicated samples ± SD. Different letters refer to significant differences at $p \leq 0.000$.

Figure 2 shows the toxicity of the anise oil at different times against adult females of *T. urticae*. The recorded LC_{50} and LC_{90} values of the anise oil after 72 h were 20.94 and 35.80 µL/L, respectively; the values after 48 h were 21.73 and 39.99 µL/L, respectively; and the values after 24 h were 22.32 and 43.98 µL/L, respectively. These data revealed that the adult females proved to be more susceptible to the toxic action of anise oil after 72 h, followed by 48 h and 24 h. There was an inverse relationship between the LC_{50} and LC_{90} values and the toxicity of the anise oil.

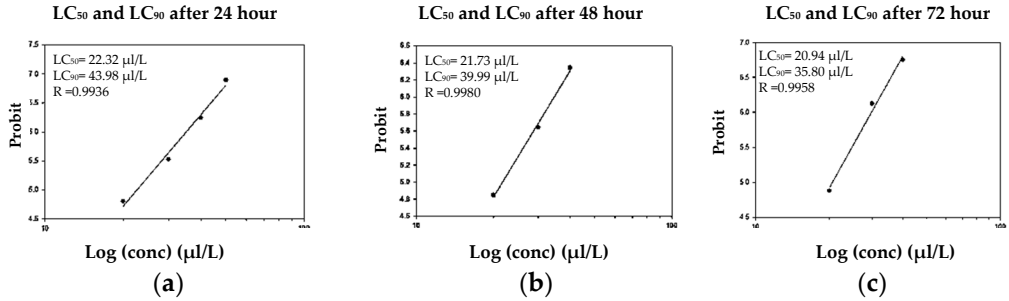
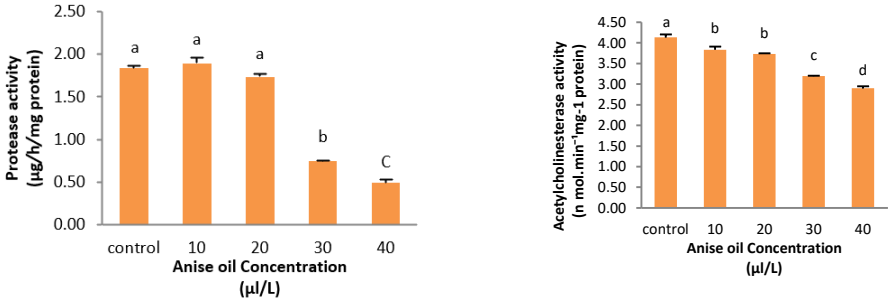


Figure 2. Toxicity lines of anise oil on adult females of *T. urticae* after 24, 48, and 72 h under laboratory conditions. (**a**) Toxicity lines after 24 h; (**b**) toxicity lines after 48 h; (**c**) toxicity lines after 72 h.

The data in Figure 3 show the effect of the anise oil on the protease and AChE activities of the two-spotted spider mites. The results showed that treatment with a low concentration of anise oil (10 µL/L) led to the activation of protease activity by about 2.26 µg/h/mg protein, and that treatment with 20 µL/L of anise oil did not cause a significant change in protease activity compared with the untreated control. On the other hand, treatment with higher dose of the anise oil, that is, 40 µL/L, led to a significant reduction in protease activity by 0.49 µg/h/mg protein. An experimental result (see Figure 3) indicated the AChE activity of *T. urticae* after 48 h of exposure to various concentrations of anise oil. The results clearly indicated that anise oil significantly inhibited AChE in a dose-dependent manner. The inhibitory effect of a low dosage of anise oil (10 µL/L) on *T. urticae* AChE was only about 7.3%. The highest reduction of AChE activity was recorded

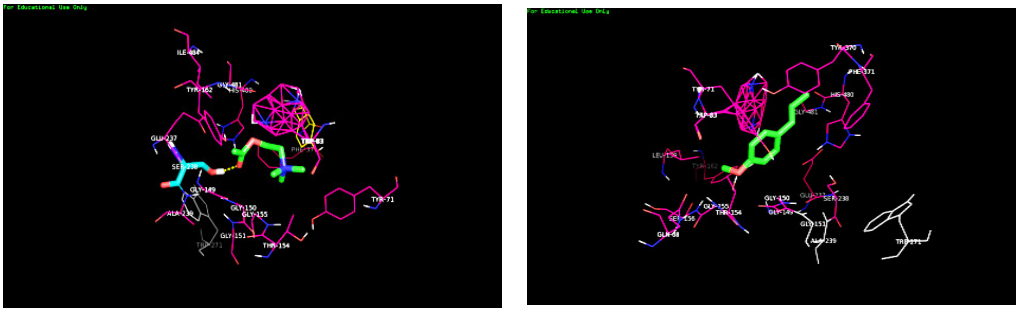
after 48 h of treatment with 40 µL/L of anise oil; the AChE activity decreased by 34% compared with the untreated control.



(**a**) (**b**)

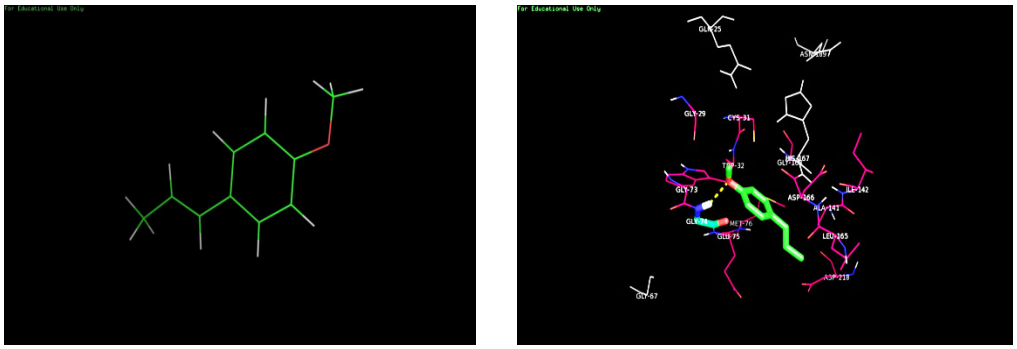
Figure 3. Enzyme activities of *T. urticae* subjected to different doses of anise oil. (**a**) Protease activity; (**b**) acetylcholinesterase (AChE) activity and for each parameter, the mean values ± SD followed by a different letter are significantly ($p \leq 0.05$) different according to LSD.

To study the effect of anise oil on AChE and protease, the main compound in anise oil was selected. Docking was performed with Autodock 4 [29]. Docking of anethole into the active site of AChE (6XYU) [22] showed the presence of hydrophobic interactions between anethole; two catalytic residues, His480 and Ser238; three residues of the oxyanion hole, Gly150, Gly151, and Ala239; and the choline-binding pocket Trp83 in the active site of the enzyme. The structures of complexes of AChE with an anethole inhibitor show the importance of these aromatic residues in ligand binding (Figures 4 and 5). The docking of anethole into the active site of cathepsin L (3F75), as one of the protease enzymes in *T. urticae*, showed the formation of a hydrogen bond between anethole and one residue, Gly74, and hydrophobic interactions between anethole and two catalytic residues, Cys31 and His167, at the active site of the enzyme (see Figure 4).



(**a**) (**b**)

Figure 4. Structures of the complex of AChE with substrate acetylecholine (**a**) and updated (**b**) structures of the complex of AChE with E–anethole (major compound in anise oil). The docking was performed with Autodock. The ligands are represented as sticks in green color; the residues that interacted with the ligand hydrophobicly are represented as lines in pink color; residues that interacted with the ligand with hydrogen bonds are represented as sticks in teal color; non-interactive residues are represented as lines in grey and hydrogen bbonds are represented as yellow dashes.



(**a**) (**b**)

Figure 5. Structures of E–anethole (**a**) and updated (**b**) structures of the complex of Cathepsin L with E–anethole (major compound in anise oil). the ligands are represented as sticks in green color; the residues that interacted with the ligand with hydrophobicly are represented as lines in pink color; residues that interacted with the ligand with hydrogen bonds are represented as sticks in teal color; non-interactive residues are represented as lines in grey and hydrogen bonds are represented as yellow dashes.

4. Discussion

In recent years, natural pesticides have become important as alternatives to synthetic pesticides due to the adverse effects of chemical control not only on environment wildlife but also on human health [33]. A lot of studies have been done on the possibility of using natural compounds in plants as an alternative to synthetic pesticides [34,35]. Studies have focused on natural materials that do not add toxic substances to the environment, decompose in a short period of time, and do not cause soil and water pollution [36]. There have been many studies on the effects of extracts from plants obtained by different methods on *T. urticae* [37]. Several essential oils and plant extracts have insecticidal properties including mites [38]. Promising results have been obtained in studies using plant extracts and essential oils in the control of pest mites. Thus, for *T. urticae*, in trials with spraying on females, *Mentha spicata* X suaveolens aqueous extract caused the death of 96% of females after 120 h of application [39]. oEssential oil of *Cymbopogon citratus* Boisduval in non-contact diffusion bioassay with oil application in filter paper in a closed container provided 100% mortality at a dose of 19×10^{-3} µL/mL of air [40].

The variability in the volatile components of anise oil in our results appears to be largely due to the stage of harvest and seasonal and environmental factors, as well as the method of extraction. In anise oil, the main compound was *E*-anethole, followed by estragole. Similarly, *trans*-anethole was previously reported as a major component of *P. anisum* [41,42]. These results are in agreement with previous investigations on anise essential oil [43,44]. Muthanna and Hiyam [45] identified *trans*-anethole as a major component of *P. anisum* essential oil (26.97%), followed by estragole (20.50%). Haşimi et al. [46] determined that the main components of anise essential oil were *trans*-anethole (52.94%), followed by isoanethole (13.89%), caryophyllene oxide (8.55%), and caryophyllene (2.4%). Mohammed et al. [47] reported that essential oil had a percentage of *trans*-anethole of 55.491%.

Anise oil showed the highest activity at a concentration of 50 µL/L. It was the least effective radical scavenger at a concentration of 10 µL/L. This means that by increasing the concentration of the essential oil, the antioxidant activity was increased. This may be due to the increase in terpenes and active substances, which have a free radical scavenging effect. The antioxidant activity of spices and herbs is attributed to the presence of volatile oils and bioactive components [48–50].

According to [51,52], anise oil possesses antioxidant properties. This is due to the fact that the seeds are rich in important minerals and compounds such as anethole, anisaldehyde, anise alcohol, acetophenone, pinene, limonene, and glycerol.

This work has shown that anise oil has acaricidal activity, possibly due to the active substances present in the essential oil. The acaricidal activity assessment reported that anise oil at 40 μL/L could fully control the two-spotted spider mites, with a mortality rate after 72 h of 96.0%. The highest concentration of anise oil caused the highest mortality rate for *T. urticae* after 24 h. The lowest mean number of eggs hatched at a high concentration of 40 μL/L was recorded during the fifth and sixth days. This may be attributed to the increase in the concentration of active substances, which also increased their effects on egg hatching. The two-spotted spider mite can be controlled with essential oils which possess odor-producing compounds including monoterpenes, sesquiterpenes, phenols, oxides, esters, aldehydes, and ketones [53]. Plants use these active compounds to protect against various arthropods such as insects and mites [54]. Vinicius et al. [5] reported that the most promising extracts for *T. urticae* control were *Origanum vulgare*, *Matricaria chamomilla,* and *P. anisum;* all produced a mortality rate above 75% in two replicates. The volatiles emanating from the essential oil, especially carvacrol, borneol, cineol, terpineol, and terpinene, have an acaricidal effect on *T. urticae* females, reaching a mortality rate of 100% as a function of concentration and exposure time [55]. In this study, a mortality rate of 96.0% was obtained when large amounts of essential oils were used. High mortality rates have been reported before; for example, Tunc and Sahinkaya [56] reported a 100% mortality rate of *T. cinnabarinus* and *Aphis gossypii Glover* when essential oils of *Cuminum cyminum, P. anisum,* and *Origanum syriacum* were used in greenhouse conditions. In addition to being toxic against the postembryonic stage of insects and mites, some essential oils have oviposition-deterring activities; for example, three essential oils extracted from *Laurus nobilis, Myrtus communis*, *P. anisum,* and *Artemisia absinthum* were toxic against the adults and eggs of *T. cinnabarinus* under laboratory conditions [57].

The ways essential oils work vary with their neurotoxicity. This confirms why anise oil inhibits the AChE growth in insects, degrading the waxy layers of insect cuticles, which obstruct digestive enzymes such as protease and inhibit glutathione-S-transferase (GST) [58]. Ahmed et al. [58] stated that the compounds (−)-terpinen-4-ol and γ-terpinene were two main ingredients with insecticidal properties and affected insect enzymes such as AChE and GST. This confirms the results of our study; anise seed oil affected various biological parameters of *T. urticae* such as AChE and protease. Results showed that this oil had a high degree of toxicity against the nervous system, as it inhibited AChE, and the highest inhibition was at a concentration of 40 μL/L after 48 h. This is related to the essential oil's high concentration of *E*-anethole, which binds to the active site of AChE by hydrophobic interactions (see Figure 3). These results are in agreement with the findings of Ivanov et al. [59], who reported that anise hyssop essential oil showed an inhibitory effect on AChE activity where the IC50 value equaled 19.25 mg/L. Anise oil concentrations of 22.32, 21.73, and 20.94 ppm led to a mortality rate of 50% of *T. urticae* mites after 24, 48, and 72 h, respectively. The obtained LC_{50} and LC_{90} values of anise oil reflect high acaricidal activity of anise oil against *T. urticae*. Rania [60] found insecticidal activity of lupine extract, olive oil, marjoram oil, anise oil, and orange oil against two strains of *A. gossypii* and *Rhopalosiphum maidis* (Fitch). Mead [61] stated that the toxic effect of *C. citratus* oil against *T. urticae* was undoubtedly due to its component citral (which has two isomers, geranial and neral). The AChE inhibitory activity is due to synergistic and antagonistic interactions between the chemical constituents of anise oil and AChE. The major compound in anise hyssop essential oil was estragol. This compound was reported to have a high ability to inhibit AChE (IC50 0.337 μmol), followed by eugenol (IC50 40.32 μmol) [62]. The results of our study indicated that anise oil affected the activity of protease; the exposure of *T. urticae* adults to anise oil at concentration of 40 μL/L for 48 h had a significant effect on the protease enzyme compared with the control. This may be attributed to the binding of *E*-anethole to the active site of the enzyme by a hydrogen bond in the residue Gly74 and the hydrophobic interactions between anethole and the catalytic residues Cys31 and His167 at the active site of the enzyme (Figure 4). On the other hand, the treatment of *T. urticae* with a low concentration of anise oil (10 μL/L) did not cause

inhibition of the protease activity. Accordingly, it can be concluded that the increase in the anise oil concentration had a paradoxical impact on the protease activity. In addition to the above findings, effective compounds have been identified in the essential oils of thyme and anise and synergistic activity has been shown [63]. Hummelbrunner and Isman [64] reported that monoterpenes produce synergistic insecticidal effects. The Chaubey [65] study reported that α-pinene and β-caryophyllene in a binary combination showed synergy, reduced the egg-laying capacity, and prevented pupation and adult emergence in *Tribolium castaneum*. These results reported earlier clearly support the results of the current study. In the current research, anise oil (*P. anisum*) was more effective against *T. urticae*. The presence of bioactive components in anise oil provides hope for the development of new natural insecticides that would be economically and environmentally sound for the management of insect pests that affect stored products. The present findings confirmed the findings of Athanase and Fedai [66] and Ahmed et al. [58]. Gas chromatography analysis for volatile oils showed that bisabolol oxide A (44.34%), carvone (70.29%), linalool (85.60%), and camphor (54.36%) were the main components of chamomile, spearmint, coriander, and rosemary volatile oils, respectively, and they may be responsible for controlling *T. urticae*. Anise oil contains most of these active substances, and it had the same effect on *T. urticae.* In addition to the effect of the main compound, *E*-anethole, on the inhibition of AChE and protease enzymes. The inhibition was competitive for the active site.

5. Conclusions

In conclusion, anise oil caused high mortality rates in *T. urticae* mites at different times with a concentration of 40 µL/L. The lowest mean number of eggs hatched was recorded at the high concentration of 40 µL/L on the fifth and sixth days of the study. The results showed that the activity of AChE was significantly inhibited at 40 µL/L, followed by 30 µL/L. This is related to the essential oil's high concentration of *E*-anethole, which binds to the active site of the AChE enzyme by hydrophobic interactions. The activities of protease decreased when mites were treated with 30 or 40 µL/L of anise oil. This is related to the essential oil's high concentration of active compounds such as *E*-anethole, –(e)-isoeugenol, limonene, linalool, and α-pinene. Therefore, we suggest that tested anise oil can be used to control mites on green bean seedlings as an alternative to harmful insecticides. Due to the devastating effects of synthetic insecticides on humans and the environment, the use of anise oil as a natural pesticide is advised.

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