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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Inhibition Effect of Some Plant Extracts on *Calliteara pudibunda* (Linneaus, 1758) Acetylcholinesterase

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Abstract: In pest control management, acetylcholinesterase inhibition is one of the important methods. Acetylcholinesterase is the target point of nerve gases and insecticides due to its important role in the nervous system. This study determined the optimum conditions of "*Calliteara pudibunda* acetylcholinesterase" and investigated some of its kinetic properties. Acetylcholinesterase inhibition studies were carried out with known inhibitors of acetylcholinesterase such as tacrine, edrophonium chloride, cypermethrin and aqueous extracts of olive leaf, walnut leaf, walnut shell, cherry laurel leaf and alder leaf. The V_{max} and K_m values of acetylcholinesterase, which showed maximum activity at 40.0 ºC and pH 7.0, were determined as 1.7±0.2 EU and 0.18±0.02 mM, respectively. In inhibition studies, the IC_{50} values of tacrine, edrophonium chloride and cypermethrin were found to be 6.5±0.2, 2.8± 0.3 and 6.0±0.8 µM, respectively. The IC_{50} values of aqueous extracts of olive leaf, alder leaf, cherry laurel leaf, walnut shell and walnut leaf were found to be 1.8± 0.2, 1.8±0.4, 1.9±0.4, 2.8±0.6 and 5.8±1.2 µg dry matter/mL, respectively. In addition, the plant extracts oleuropein and phenolic substance amounts were determined and correlated with IC_{50} values. As a result, these plant extracts used in the study can be recommended as an alternative biopesticide source to control such pests through acetylcholinesterase inhibition.

Keywords: Acetylcholinesterase, biopesticides. *Calliteara pudibunda*, inhibition, pest management.

Bazı Bitki Ekstraktlarının *Calliteara pudibunda* (Linneaus, 1758) Asetilkolinesteraz Üzerindeki İnhibisyon Etkisi

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Öz: Zararlı mücadele yönetiminde asetilkolinesteraz inhibisyonu önemli yöntemlerden biridir. Asetilkolinesteraz sinir sistemindeki önemli rolü nedeniyle sinir gazlarının ve böcek ilaçlarının hedef noktasıdır. Bu çalışmada, *Calliteara pudibunda* asetilkolinesterazının optimum koşulları belirlenmiş ve bazı kinetik özellikleri incelenmiştir. Asetilkolinesteraz inhibisyon çalışmaları, asetilkolinesterazın bilinen inhibitörleri olan takrin, edrofonyum klorür ve sipermetrin ile zeytin yaprağı, ceviz yaprağı, ceviz kabuğu, kiraz defnesi yaprağı ve kızılağaç yapraklarının sulu ekstraktları ile yürütülmüştür. 40.0 ºC ve pH 7.0'de maksimum aktivite gösteren asetilkolinesterazın, V_{max} ve K_m değerleri sırasıyla 1.7±0.2 EU ve 0.18±0.02 mM olarak belirlenmiştir. İnhibisyon çalışmalarında kullanılan takrin, edrofonyum klorür ve sipermetrinin IC_{50} değerleri sırasıyla 6.5±0.2, 2.8±0.3 ve 6.0±0.8 µM olarak bulunmuştur. Zeytin yaprağı, kızılağaç yaprağı, kiraz defnesi yaprağı, ceviz kabuğu ve ceviz yaprağının sulu ekstraktlarının IC_{50} değerleri sırasıyla 1.8±0.2, 1.8±0.4, 1.9±0.4, 2.8±0.6 ve 5.8±1.2 µg kuru madde/mL olarak bulunmuştur. Ayrıca bu bitki ekstraktlarının oleuropein ve fenolik madde miktarları belirlenerek IC_{50} değerleri ile ilişkilendirilmiştir. Sonuç olarak çalışmada kullanılan bu bitki ekstraktlarının asetilkolinesteraz inhibisyonu yoluyla bu tür zararlıların kontrolünde alternatif biyopestisit kaynağı olarak önerilebileceği düşünülmektedir.

Anahtar kelimeler: Asetilkolinesteraz, biyopestisitler. *Calliteara pudibunda*, inhibisyon, haşere yönetimi.

INTRODUCTION

Calliteara pudibunda (Linneaus, 1758) (Lep.: Erebidae, Lymantriinae), a Lepidoptera species, is a leaf-consuming pest known as the beech pest (Sarıkaya et al., 2021). Since 1810, outbreaks of this pest have been reported in beech and oak forests in many European countries, including Germany, Sweden, Denmark, Poland, Romania, and Ukraine (Mazzoglio et al., 2005). Its larvae are herbivorous and feed primarily on *Fagus* and *Carpinus*, but also on other deciduous trees, herbaceous plants, and it can cause defoliation of trees (Göktürk & Aksu, 2005; İpekdal, 2022; Mazzoglio et al., 2005; Sarıkaya et al., 2021;

Trofimova, 2012). In our country, pest species cause epidemics or economic damage are not frequently in beech forests. However, it was determined that *Calliteara pudibunda (C. pudibunda*), which was reported to cause damage to alder and birch trees in the Artvin region in 2005 (Göktürk & Aksu, 2005), caused an epidemic in the beech forests located on the Bursa-İnegöl and Kütahya-Domaniç borders in 2019, damaging an area of 453.80 hectares (Sarıkaya et al., 2021). This pest, which was detected in Artvin and Bursa provinces in Turkey, has also been reported to be detected in Balıkesir, Düzce, Giresun, Hatay, İstanbul, Kütahya, Malatya, Sakarya, Samsun and Yalova provinces (İpekdal, 2022; Ipekdal & Avcı, 2023; Öztürk et al., 2024). According to Sarıkaya (2019), the pest is likely to expand its distribution in our country, especially in the Marmara and Black Sea regions. It has been reported that the fact that the pest feeds polyphagously on other forest trees may pose a significant potential threat to forests (Sarıkaya, 2019).

The forest ecosystem, which is the most basic and indispensable part of human life, vital to importance for humanity in terms of ecosystem services such as carbon sequestration, forest products, biodiversity, and climate change (Akyol & Sarıkaya, 2017; Ding & Eldridge, 2024). Insects, which have a natural role in the forest ecosystem, can multiply excessively for various reasons, including human impact, and can disrupt the balance of the ecosystem by causing damage to forests. For the ecosystem to be sustainable, it is of great importance to keep the factors that make up the ecosystem in balance, protect them, and take the necessary precautions to return them to their natural balance (Akyol & Sarikaya, 2017; Akyol & Tolunay, 2014).

Acetylcholinesterase (AChE; E.C. 3.1.1.7), found in many conductive tissues, especially nerve, and muscle tissues is a cholinergic enzyme called acetylcholine acetylhydrolase (Duranay et al., 2019; Ramesh et al., 2018). AChE, which plays an important role in neurotransmission, rapidly hydrolyzes the neurotransmitter acetylcholine (ACh) into acetate and choline (~25000 ACh molecules per second) to complete neurotransmission. Because of the important role of AChE in the nervous system, it is the target point of nervous system diseases, nerve gases, and insecticides (Colovic et al., 2013; Wang et al., 2022). The decrease or termination of AChE can lead to nervous system disorders such as nerve overstimulation or blocking of neurotransmission, even paralysis, and death (Colovic et al., 2013; Dinçer & Akpınar, 2023; Dinçer & Kizil, 2022; Li et al., 2021; Soto-Mancera et al., 2020). Compounds that can reduce or completely stop AChE activity are called AChE inhibitors or anticholinesterases (Colovic et al., 2013; Rampa et al., 2000). AChE inhibitors can be divided into two groups according to their mode of action: reversible and irreversible. Reversible inhibitors, which can be competitive or non-competitive, are mostly used in therapeutic applications, irreversible inhibitors have been reported to have toxic effects (Colovic et al., 2013; Li et al., 2021). Partial inhibition of AChE activity in the brain by AChE inhibitors that cross the blood-brain barrier increases endogenous acetylcholine levels, which is useful in the symptomatic treatment of diseases such as myasthenia gravis, glaucoma, dementia, and Alzheimer's disease. However, complete inactivation of AChE, which can occur with organophosphate chemical warfare agents such as nerve gases and insecticides, can lead to excessive ACh accumulation, resulting in paralysis or death (Li et al., 2021; Patočka et al., 2004). Because of this important role of AChE in the nervous system, pest control studies have focused on AChE inhibition (Dinçer & Akpınar, 2023; Dinçer & Kızıl, 2022; Gao et al., 1998; Keane & Ryan, 1999; Li & Han, 2002; Mohamed et al., 2020). AChE studies in both mammals and insects have generally used AChE obtained from body tissues or whole-body homogenates due to their proximity to the cellular environment (Keane & Ryan, 1999; Li & Han, 2002; Moores et al., 1994).

Organophosphates, pyrethrins, and carbamates, which are among the pesticides widely used in agriculture, have toxic effects and the presence of their residues in air, food, groundwater, water, and soil has become a major problem for the environmental and health concern (Dinçer & Akpınar, 2023; Dinçer & Kızıl, 2022; Farag et al., 2021; Li et al., 2021; Mdegela et al., 2010; Poirier et al., 2017; Pundir & Chauhan, 2012; Vinotha Alex & Mukherjee, 2021). The residues of these pesticides, which can be mutagenic, can reach living organisms and accumulate in food chains, even damaging their genetic structures (Aydogdu et al., 2017; Aydoğdu & Güner, 2012). Their toxicity is based on the inhibition of AChE, which is necessary for the functioning of the central nervous system. Organophosphate and carbamate pesticides, which bind to the serine residue in the active site of AChE with a covalent bond, inhibit AChE and cause the accumulation of the neurotransmitter acetylcholine. This disrupts the transmission of electrical nerve impulses at synapses in the nervous system, causing respiratory and myocardial failures, paralysis, and even death of the insect (Abou-Donia, 2003; Colovic et al., 2013; Pundir & Chauhan, 2012; Soto-Mancera et al., 2020). Cypermethrin, one of the pyrethrins widely used in agricultural, veterinary, and domestic pest management, acts as a fast-acting neurotoxin in insects. Even low concentrations of this pesticide have been reported to have toxic effects on the brains of laboratory animals, many fish, and aquatic invertebrates (Farag et al., 2021; Prusty et al., 2015; Singh et al., 2014). Considering these negative effects on humans and animals, it is clear that the use of such pesticides to control forest pests would not be appropriate from a health, environmental and thus ecosystem

perspective. In order to eliminate these negative effects, alternative natural herbal products that are safe, environmentally friendly and inexpensive as an alternative to pesticides are attracting attention and being researched (Dinçer & Akpınar, 2023; Dinçer & Kizil, 2022; Poirier et al., 2018; Rosell et al., 2008).

The aim of this study was to investigate alternative biopesticides that could be used instead of pesticides in the control of *C. pudibunda*, which causes extensive damage to beech forests. For this purpose, the inhibitory effects of tacrine, cypermethrin, and edrophonium chloride which are AChE specific inhibitors, and aqueous extracts of olive leaf, walnut leaf, alder leaf, cherry laurel leaf and walnut shell on *C. pudibunda* AChE were investigated.

MATERIAL AND METHOD

Materials: The chemicals used in the study were purchased from Sigma-Aldrich Company. *Calliteara pudibunda* (Linneaus, 1758) samples was collected from pure beech and beech-oak mixed stands at an average altitude of 500 m in the Şahmelek neighbourhood of the Karacabey district of Bursa province, Turkey, and stored at -20 ºC. Olive leaves (*Olea europaea sativa* L.), walnut leaves (*Juglans regia* L.), and walnut shells (*Juglans regia* L.) were collected from Bursa, cherry laurel leaves (*Laurocerasus officinalis* L.), and alder leaves (*Alnus glutinosa* subsp. barbata) were collected from Trabzon. These leaves dried at room temperature were ground to powder using a grinder and then passed through a 60-mesh sieve to use for in extraction.

Methods:

Crude Extract Preparation: Approximately 5 grams of *Calliteara pudibunda* samples was homogenised in 20 mL of 50 mM pH 7.4 sodium phosphate buffer containing 0.5% Triton X-100 and 1 mM EDTA in an ice bath. The supernatant obtained by centrifugation of the homogenate at 20,000xg for 45 minutes at 4°C was filtered through a syringe filter unit with a pore size of 0.45 µm and used as the crude extract (Dinçer & Akpınar, 2023; Dinçer & Kızıl, 2022; Son et al., 2002).

Enzyme Assay: AChE activity was determined spectrophotometrically in the presence of acetylthiocholine iodide (ATC) substrate (Ellman et al., 1961). The volume of the mixture consisting of 1.5 mM ATC, 0.2 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and enzyme solution was filled up to 1 mL with 0.1 M (pH 8.0) phosphate buffer. The change in absorbance of the yellow compound formed as a result of the enzymatic reaction was recorded at a wavelength of 412 nm for 20 minutes. The amount of enzyme that converts 1.0 µM of substrate into the product in one minute at 25 ºC under optimal conditions is one enzyme unit (EU) (Cavdar et al., 2019; Dinçer & Kızıl, 2022; Ellman et al., 1961; Son et al., 2002). The kinetic parameters, Michaelis–Menten constant (K_m), and maximal enzyme velocity (V_{max}) were calculated using Lineweaver and Burk plots (Lineweaver & Burk, 1934).

Oleuropein Analysis by High-Performance Liquid Chromatography (HPLC): High-performance liquid chromatography was used to determine the oleuropein content of plant extracts. The chromatographic method conditions applied in HPLC were prepared according to Ansari et al (Ansari et al., 2011). Standard oleuropein standard solutions were prepared at different concentrations ranging from 0.005 to 0.5 mM. The peak areas of these solutions at retention times were determined and a calibration graph was drawn. The oleuropein contents of plant extracts was determined by HPLC analysis using this calibration graph.

Determination of Total Phenolic Content: The total phenolic content of the plant extracts was determined using the Folin-Ciocalteu reagent at 760 nm. The calibration curve was established with aqueous solutions of gallic acid prepared at different concentrations ranging from 0.005 to 0.5 mM. The change in absorbances with gallic acid solutions and plant extracts was measured at 760 nm. The results were expressed as Gallic Acid Equivalent (GAE) determined by the regression equation of the calibration curve (Hayouni et al., 2007; Lister & Wilson, 2001).

AChE Inhibition: AChE inhibition studies were carried out in the presence of ATC substrate under optimal conditions. The inhibitor concentration that reduced the enzyme activity by 50% was determined as the IC_{50} value. Solutions of tacrine, edrophonium chloride, and cypermethrin known as specific inhibitors of AChE, were prepared in the concentration ranges 1-50 µM (Dinçer & Kızıl, 2022; Mohamed et al., 2020). The plants were dried in the shade and subsequently pulverised using a grinder. Approximately 1.2 g of dry plant leaf powder was extracted in 20 mL of distilled water in a shaking water bath for 24 hours and centrifuged at 10,000xg for 30 minutes. The solvents of the obtained solutions were removed by evaporator at 60 °C under reduced pressure. The remaining dry substances were scraped with a spatula and weighed with a precision of 0.0001. Distilled water solutions with a concentration range of 1-100 µg dry matter/mL were prepared from the quantified dry matter and used in inhibition studies. (Dinçer & Akpınar, 2023; Dinçer & Kızıl, 2022; Acet, 2019). The inhibitory effects of all the prepared inhibitor solutions on the AChE activity of *C. pudibunda* were determined, and the IC_{50} values of AChE were calculated by plotting the percentage inhibition graphs against the inhibitor concentrations.

RESULTS AND DISCUSSION

The AChE activity of *C. pudibunda* was found to be optimal at 40.0±0.1 °C and pH 7.00±0.05. It was observed

that AChE of *C. pudibunda* lost its activity by approximately 42% - 60% between pH 4.0 and 6.0 and by approximately 18% - 68% between pH 8.0 and 10.0. It can be said that this ACHE is quite sensitive to pH (Table 1, Fig 1). The optimum conditions are consistent with the optimum conditions of many AChEs in the literature (Dinçer & Akpınar, 2023; Dinçer & Kızıl, 2022; Duranay et al., 2019; Meng et al., 2016; Mohamed et al., 2020; Prabhakaran & Kamble, 1996; Shi & Zhang, 1981).

Table 1. Kinetic parameters of *C. pudibunda* AChE.

Optimal pH	Optimal Temperature (ºC)	*Vmax* (EU)	K_m (mM)
7.00±0.05	40.0±0.1	1.7±0.2	0.18±0.02

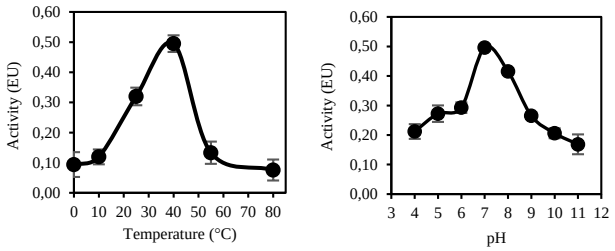

Fig 1. The graphs of *C. pudibunda* AChE activity versus temperature and pH.

AChE kinetic parameters of *C. pudibunda* in the presence of ATC were determined by the Lineweaver-Burk plot. The V_{max} and K_m values for the hydrolysis of ATC used as a substrate were determined as 1.7±0.2 EU and 0.18±0.02 mM, respectively (Table 1 and Fig.2).

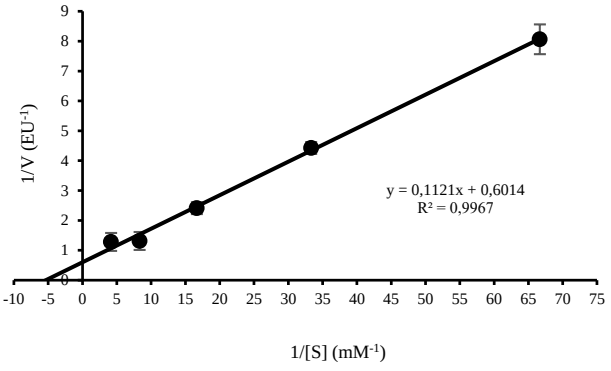

Fig 2. The Lineweaver-Burk plot of *C. pudibunda* AChE.

The K_m value of *C. pudibunda* AChE was found to be greater than the K_m values of *Halyomorpha halys* (0.02±0.006 mM) (Dinçer & Akpınar, 2023), *Ricania simulans* adult (0.04±0.01 mM), and *Ricania simulans* nymph (0.02±0.01 mM) AChEs (Dinçer & Kızıl, 2022) and is smaller than the K_m values of *Mytilus galloprovincialis* (1.3 mM) (Duranay et al., 2019), *Scomberomorus niphonius* (0.311 mM) (Zhu et al., 1993), and *Heterorhabditis bacteriophora* (0.27 mM) (Mohamed et al., 2007). The K_m value of AChE in *C. pudibunda* was found to be nearly to the K_m values of AChE from *Oreochromis aurea* (0.183 mmol/L) (Ding et al., 2011), *Pseudosciaena crocea* muscle (0.125 mmol/L) (Dong, 1995), and *Nebia albiflora* muscle (0.10 mM) (Shi & Zhang, 1981). The V_{max} value of the AChE of *C. pudibunda* (1.7 EU) was found to be close to the V_{max} values of the AChEs of *Halyomorpha halys* (0.99 EU) (Dinçer and Akpınar, 2023), *Ricania simulans* adult (1.2 EU) (Dinçer and Kızıl, 2022) and *Ricania simulans* nymph (0.9 EU). It is lower than the V_{max} values given for *Nebia albiflora* muscle (100 EU) (Shi & Zhang, 1981), and *Pseudosciaena crocea* muscle (125 EU) (Dong, 1995).

The IC_{50} values of the competitive AChE inhibitors tacrine and edrophonium chloride used in the inhibition studies were determined to be 6.5±0.2 and 2.8± 0.3 µM, respectively. In addition, the IC_{50} value of cypermethrin, which is widely used in pest control, was found to be 6.0±0.8 µM. It was observed that the known inhibitors were effective against *C. pudibunda* AChE (Table 2). In the inhibition study of AChE purified from *Halyomorpha halys*, the IC_{50} value of cypermethrin was determined to be 9.2+0.5 µM (Akpınar, 2024). In the literature, when tacrine and edrophonium chloride were used as inhibitors, IC_{50} values for AChEs obtained from different sources were reported as 0.08 ± 0.003, and 15.0 ± 1.0 µM for *Halyomorpha halys* (Dinçer & Akpınar, 2023),18.0 ± 1.9, and 2.4 ± 0.3 µM for *Ricania simulans* adults, 1.2 ± 0.4, and 0.6 ± 0.09 µM for *Ricania simulans* nymphs (Dinçer & Kızıl, 2022), and 9.16 and 0.68 µM for *Electrophorus electricus* (Mutunga et al., 2009), respectively. In the inhibition study of AChE of German cockroach (Blattella germanica) with tacrine, the IC_{50} value was found to be 68 nM (Mutunga et al., 2009). The IC_{50} values obtained from the inhibition of AChEs are seen to be consistent with the data reported in the literature. The fact that these specific inhibitors are effective against *C. pudibunda* AChE activity is evidence that the enzyme used in the study is AChE.

Table 2. IC_{50} values of the AChE of *C. pudibunda* in the presence of ATC.

Substance	IC_{50}	OLE Concentration of the plant leaves extracts (mM)	Total Phenolic Amount GAE) (mM)
Tacrine	6.5±0.2 µM	-	-
Edrophonium chloride	2.8± 0.3 µM	-	-
Cypermethrin	6.0±0.8 µM	-	-
Olive leaf aqueous extract (*Olea europaea sativa L.*)	1.8± 0.2 µg dry matter/mL	0.27±0.05	1.7±0.3
Alder leaf aqueous extract (*Alnus glutinosa* subsp. Barbata)	1.8±0.4 µg dry matter/mL	0.25±0.08	1.6±0.4
Cherry laurel leaf aqueous extract (*Laurocerasus officinalis* L.)	1.9±0.4 µg dry matter/mL	0.09±0.04	1.2±0.2
Walnut shell aqueous extract (*Platanus orientalis* L.)	2.8±0.6 µg dry matter/mL	0.09±0.02	1.0±0.3
Walnut leaf aqueous extract (*Juglans regia* L.)	5.8±1.2 µg dry matter/mL	0.08±0.03	0.9±0.4

Aqueous extracts of plants were used in inhibition studies on *C. pudibunda* AChE and the highest inhibition effect were observed in the olive leaf (IC_{50}:1.8±0.2 µg dry matter/mL) and alder leaf extract (IC_{50}:1.8±0.4 µg dry matter/mL), followed by cherry laurel leaf (IC_{50}:1.9±0.4 µg dry matter/mL), walnut shell (IC_{50}:2.8±0.6 µg dry matter/mL), and walnut leaf (IC_{50}:5.8±1.2 µg dry matter/mL) (Table 2). The IC_{50} value of the olive leaf extract used in this study for AChE in *C. pudibunda* was determined to be lower than the IC_{50} values of *Halyomorpha halys* (20.3 ± 0.9 µg dry matter/mL) (Dinçer & Akpınar, 2023), *Ricania simulans* adults (20.3 ± 1.2 µg dry matter/mL), and *Ricania simulans* nymphs (16.2 ± 0.8 µg dry matter/mL) (Dinçer & Kizil, 2022). In addition, the IC_{50} values reported for alder leaf and walnut leaf extracts are lower than those reported for *Halyomorpha halys* (IC_{50} for alder leaf: 19.0 ± 1.7 µg dry matter/mL, and IC_{50} for alder leaf: 108.0 ± 40 µg dry matter/mL) (Dinçer & Akpınar, 2023). It was observed that the IC_{50} values in this study were consistent with other data in the literature, and as can be understood from the literature, differences in the IC_{50} values of the inhibitors used are observed by changing the source of the AChE enzyme.

Oleuropein concentrations of aqueous plant extracts were determined according to the equation (y=785.96x; R^2=0.99) obtained from the oleuropein standard calibration. It was calculated as 0.27±0.05 mM in olive leaf, 0.25±0.08 mM in alder leaf, 0.09±0.04 mM in cherry laurel leaf, 0.09±0.02 in walnut shell, and 0.08±0.03 mM in walnut leaf (Table 2). The total phenolic contents of aqueous plant extracts was determined according to the equation obtained from the calibration curve drawn with gallic acid standard (y = 2.1454x; R^2 = 0.99) and the results were expressed as GAE. The total phenolic content of olive leaf, alder leaf, cherry laurel leaf, walnut shell, and walnut leaf extracts were determined to be 1.7±0.3 mM, 1.6±0.4 mM, 1.2±0.2 mM, 1.0±0.3 mM, and 0.9±0.4 mM, respectively (Table 2). Many local plant compounds used pest control are known to have an inhibitory effect on the AChE of insects (Abdellaoui et al., 2019; Grdiša & Gršić, 2013; Gülçin et al., 2020). It has been reported that polyphenols may have anti-nutritional properties due to their ability to inhibit digestive proteases and hydrolases, which are detrimental to insect growth and development (Céspedes et al., 2004). Many studies have shown that olive (*Olea europaea* sativa L.) leaves exhibit strong biological activities due to their high content of phenolic compounds, and these strong effects have been identified especially with oleuropein (Ben Hamouda et al.,

2015; Dinçer & Akpınar, 2023; Dinçer & Kizil, 2022; Jemai et al., 2009; Zari & Al-Attar, 2011).

As a result, according to the data obtained in this study, the plant extracts that show the best inhibitory effect can be used periodically in forest areas where the pest is present. Ingestion of the substances in these plants, both through diet and inhalation, causes the inhibition of AChE in the pest, resulting in ACh accumulation in nerve cells. ACh, which accumulates particularly in nerve endings, causes paralysis of nerve conduction, which can lead to paralysis or death of the pest. In the context of the forest ecosystem, this recommended use allows pest populations to be controlled with aqueous plant extracts without the need for chemical pesticides.

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