International Journal of Secondary Metabolite
2022, Vol. 9, No. 1, 27–42
https://doi.org/10.21448/ijsm.1032863
Published at https://dergipark.org.tr/en/pub/ijsm
Research Article

Inhibitory effect on acetylcholinesterase and toxicity analysis of some medicinal plants

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Abstract: This study aimed to analyse the inhibition of different extracts of *Rosmarinus officinalis, Pistacia terebinthus* and *Sideritis dichotoma* on acetylcholinesterase enzyme of *Drosophila melanogaster*. Additionally, the biological features including antioxidant activity, phenolic contents, antibacterial effects and *in vivo* toxicities were identified using radical scavenging, Folin-Ciocalteu, disc diffusion methods, and larval (eclosion) assay using *Drosophila*, respectively. Also, GC-MS was used to determine of the terpene-derivative compositions of the plants. IC_{50} values on acetylcholinesterase were determined between 0.57±0.02-2.54±0.11µg μL^{-1} for ethanol, 0.86±0.05-2.19±0.15µg μL^{-1} for methanol and 1.98±0.13-4.76±0.24µg μL^{-1} for water extracts. Inhibition types of *Rosmarinus, Pistacia* and *Sideritis* were uncompetitive, competitive and competitive, respectively. The antioxidant activities of the extracts were between 77.87±1.72-96.94±1.84% against DPPH and 90.57±2.18-98.18±2.36% against $ABTS^+$ radicals. GC/MS results showed that carvacrol and thymol were the major monoterpenes of *Pistacia* and *Sideritis*, while limonene and borneol were the main monoterpenes of Rosmarinus. The strongest antibacterial activities were observed with *Rosmarinus* and *Sideritis* against *Staphylococcus aureus* and *Escherichia coli*, respectively with an inhibition zone larger than 15 mm. According to the *in vivo* toxicity study, all extracts were found non-toxic to *Drosophila*, and they ameliorated H_2O_2 induced decrease of puparation, survival rate and eclosion values.

ARTICLE HISTORY
Received: Dec. 05, 2021
Revised: Jan. 23, 2022
Accepted: Feb. 02, 2022

KEYWORDS
Acetylcholinesterase inhibition,
Alzheimer's disease,
Rosmarinus officinalis,
Sideritis dichotoma,
Pistacia terebinthus.

1. INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder that shows memory loss as a primary symptom and increased incidences are observed in industrialized countries having elderly populations. Although the pathogenesis of AD could not be fully elucidated, the most clarified hypothesis is the lack of the acetylcholine (ACh) molecule, known as the cholinergic hypothesis(Cavdar *et al.*, 2019). ACh molecule acts as a neurotransmitter in the synaptic gap and provides information flowing among neurons, so the cholinergic hypothesis is explained by the deficiency of acetylcholine and the loss of the cholinergic system (Adewusi *et al.*, 2011). The predominant marker of cholinergic system deficiency can be an increased activity of

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ISSN-e: 2148-6905

acetylcholinesterase (AChE) (EC 3.1.1.7), which degrades ACh, and/or the inhibition of cholineacetyltransferase, which is involved in the synthesis of acetylcholine (Fu *et al.,* 2004). The recovery of ACh can be carried out by inhibition of AChE with utilized inhibitors. Therefore, many AChE inhibition studies have been done to solve this problem. Many synthetic drugs are available on the market such as tacrine, donepezil, rivastigmine and galanthamine as AChE inhibitors (Yang *etal.,* 2015; Cavdar *et al.,* 2019;Dave *et al.,* 2000). In fact, AChE inhibitors for AD treatment are the only group of drugs in which a certain success ratio is achieved, but their use have been limited due to their detrimental side effects (Colovic, *et al*., 2013).

Another reason for the progression of AD is oxidativestress that leads to neurotoxicity through the generation and spread of reactive oxygen species (ROS)(Zhao& Zhao, 2013).Therefore, AD prevention or treatment with natural antioxidants should be considered as an alternative approach. Some medicinal plants are used as natural components of AChE inhibitors instead of synthetic drugs because of their prosperous antioxidant capacities. For example, huperzin A is a promising drug for treating AD symptoms with a very strong and reversible inhibitory effect on AChE and it is isolated from a plant, *Lycopodium serratum* (Thunb.) Trev. (Syn. *Huperzia serrata* Thunb.) (Ozarowski *et al*., 2017; Wang *et al*., 2006). In addition, there are more interesting results in the literature about the inhibitory effects of some other plant extracts like *Salvia miltiorrhiza radix* extracts which have stronger inhibitory capacities than huperzin A (Ozarowski *et al.,* 2017). Some *Salvia* species were also reported as memory enhancers because of their monoterpene compositions that lead to strong and reversible anti-acetylcholinesterase activities both *in vitro* and *in vivo* (Bahadori *et a*l., 2016; Perry *et al*., 2000). Another examples showing the advantage of strong antioxidant activities to deal with neurodegenerative diseases are *Gingko biloba* and *Panax ginseng* plants (Bastianetto *et al.,* 2000; Chang *et al.,* 2016).

In this study, *Rosmarinus officinalis* L (*RO*), *Pistacia terebinthus* L (*PT*) and *Sideritis dichotoma* Huter (*SD*) plant samples with known chemical profiles were analyzed in detail for the AChE inhibition capacities. The results were compared with the previous findings in which some of the extracts in different concentrations were found as ineffective. In addition, the antioxidant properties were revealed by DPPH and ABTS radical scavenging methods, the phenolic contents were identified by Folin-Ciocalteu method and terpenes in these plant extracts were analyzed using gas chromatography coupled to mass spectrometry (GC-MS). The antimicrobial effects of the extracts against pathogenic bacteria were also analyzed in this study by disc diffusion method because it is known that the dysbiosis of microbes, which can occur because of the pathogenic bacteria invading the intestine, may lead to brain dysfunctions and AD may be associated with that (Angelucci *et al*., 2019). Considering the potential use of these plants for therapeutic purposes, it is also necessary to better understand the toxicities in living organisms. Therefore, *in vivo* toxicities of *RO, PT* and *SD* were analyzed in this study using *Drosophila melanogaster* as a model organism because of the many developmental mechanisms they share with mammals (Macedo *et al*., 2017).

2. MATERIAL and METHODS

2.1. Materials

Rosmarinus officinalis (*RO), Pistacia terebinthus (PT)* and *Sideritis dichotoma (SD)* were collected and identified by Prof. Dr. Serap DOĞAN at their ripening period in Balikesir, Turkey. The body, leaf, flower parts and fruits of the collected plants were powdered with a grinder mill after drying at room temperature in the dark. All chemicals were purchased from Sigma-Aldrich.

2.2. Methods

2.2.1. *Preparation of Plant Extracts*

The powdered *RO, SD* and *PT* samples were prepared with MeOH, EtOH and water solvents. A 0.5 g portion of powdered samples were dissolved in5 mL solvent. It was kept in a fridge (+4°C) overnight. Then, it was centrifugated for 10 min at 4000 rpm, and supernatant was removed. After the centrifugation, the pellets were rewashed with 5 mL and 2 mL of solvents. Then, the supernatants were combined. Solvents were removed by evaporation process. The residuals were stored at -20 °C until analysis. Stock solutions of the extracts were prepared to use as 25 mg mL^{-1} for all analyzes.

2.2.2. *Preparation of Enzyme Extract*

100 mg of *D. melanogaster* larvae were homogenized by tissue homogenisator in 1mL of 50 mM phosphate buffer (pH 8.0) containing 300 mM sucrose. The homogenate was centrifuged at 4000 g for 4 min at 4 ºC. Supernatant was separated and used for experimental purposes (Assis *et al.,* 2012).

2.2.3. *Enzyme Activity and Inhibition*

AChE enzyme reaction was measured spectrophotometrically by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of thiocholines with DTNB. AChE activity and inhibition assays were performed according to the methods decribed by Ellman et al. (Ellman et al., 1961) and Senol et al. (Şenol *et al.,* 2010).

In order to determine the enzyme activity,150 µL of 0.1mM phosphate buffer (pH 8.0), 20 µL of 10 mM DTNB and 20 µL of AChE solutions were combined in a 96-well microplate with a multi-channel automatic pipette, and then incubated at 37 °C for 15 minutes. After the incubation, the reaction was started with the addition of 10 µL of 10 mM acetylcholine iodide and monitored by a microplate reader at 412 nm(Şenol *et al.,* 2010). The experiments were assayed in triplicate.

For inhibition assay, test mixtures (200 µL total volume) were prepared with 0.1 mM phosphate buffer (pH 8.0, 120-155 µL of 0.1mM), substrate solutions (ACh and DTNB) at various concentrations prepared in buffer (2.5 -22.5 µL of 10mM), the inhibitor solution (25µg μL^{-1}) at fixed concentrations and 20 µL enzymatic extract solutions. Blank (reference) sample contained all of the components except the enzyme extract with a final volume of 190 µL. The reaction was initiated by adding the substrate to the assay medium. The IC_{50} values were determined for all extracts in this way. The types of inhibition were determined using an extract of each plant sample with the best IC_{50} value. The inhibition kinetic analysis of *D. melanogaster* AChE was determined in the absence and in the presence of *RO*-EtOH, *SD*-EtOH, and *PT*-MeOH at two different concentrations. Inhibition constants (Ki and Ki') were concluded from the Lineweaver–Burk plots (Doğan*et al*., 2011).

2.2.4. *Determination of antioxidant capacities*

2.2.4.1. DPPH radical scavenging activity. The antioxidant capacities of *RO, SD* and *PT* were determined using 1,1–diphenyl-2- picrylhydrazyl (DPPH) radical scavenging activity (Blois, 1958). A 0.024 g portion of DPPH was dissolved in 100 mL MeOH. Then, 0.05mL of plant extract, 2.5 mL of DPPH solution and 2.5 mL of MeOH were added into a test tube and were kept in the dark for 1 h. For the control, MeOH was used instead of a sample. Spectrophotometric measurements were done at 517 nm. The radical scavenging activity of the samples were calculated using the following formula;

Antioxidant Activity (%) = [1- (absorbance of sample / absorbance of control)] x 100

2.2.4.2. ABTS radical scavenging activity. ABTS radical scavenging activity of the samples were performed by the method of Re et al. (Re *et al.,* 1999). $ABTS^+$ radical solution was prepared using equal volumesof 7 mM ABTS salt and 2.4 mM ammonium persulphate and kept in dark overnight. After then, the solution was diluted with MeOH until an absorbance of 1.50±0.01 at 734 nm was obtained. This absorbance was recorded as a control. For the sample analysis, 2.95 mL of the $ABTS^+$ solution and 0.05 mL of sample (extract) were added in a 3 mL cuvette. Measurements were done at 734 nm by a UV-Visible spectrophotometer (Perkin Elmer lambda-35 UV-Visible spectrophotometer). The measurements were performed in triplicate for each extract. ABTS radical scavenging activity (%) of the extracts were calculated with the following formula;

Antioxidant Activity (%) = [1- (absorbance of sample / absorbance of control)] x 100

2.2.5. *Determination of total phenolic content*

The phenolic contents of *RO, SD* and *PT* were analyzed by the Folin-Ciocalteu method (Dogan *et al*., 2010). A 3.5 mL portion of distilled water, 0.25 mL Folin reagent, and 0.25 mL of extract were combined in a test tube and incubated in the dark for 3 min at room temperature. $NaCO_3$ was added to the test tube (1 mL of 20%) and incubated for 40 min at 40 °C. For the control sample, MeOH was used instead of the plant extract. After the 40 min, absorbance values of all samples were measured at 685 nmby UV-Visible spectrophotometer. Total phenolic compounds were identified using the gallic acid calibration curve, and the results were calculated as *µg gallic acid/g*.

2.2.6. *GC-MS analysis for the composition of terpene derivatives*

The composition of the plant extracts' terpene derivatives were performed by capillary GC/MS using Shimadzu 6890N Network GC-2010 plus system combined with Shimadzu GC/MS-QP2010 ultra mass spectrometerdetector.

In order to perform GC analysis 30m x 0.25 mm x 0.25 µm HP Innowax Capillary column was used. The oven program was adjusted to keep the column'sinitial temperature at 60 ºC for 10 min after injection, rise to 220 °C with 4 ºC/min heating ramp for 10 min and increase to 240 °C with 1 ºC/min heating ramp. The injector temperature was adjusted to 250 °C, carrier gas was helium, in let pressure was 20.96 psi, linear gas velocity was 28 cm/s, column flow was 1.2 mL/min, the split ratio was 40:1 and injection volume was 1.0 µL.

MS conditions were adjusted as follows; ionization energy:70 eV; ionsource temperature: 280 °C; integral temperature: 250 ºC; and mass range: 34–450 atomic mass units. Identification of the terpenes in the *RO, PT* and *SD* were determined by comparison of their mass spectra and retention times with the GC/MS Wiley and Nist Mass Spectral Searchlibrary. The proportion of the compounds were calculated from the GC peak areas by the normalization method.

2.2.7. *Disc diffusion method for antibacterial activity*

The bacteria were maintained on Muller-Hinton agar (MHA). Two bacteria strains were selected, including the Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538) and the Gram-negative bacteria *Escherichia coli* (ATCC 8739). Antibacterial activities of the samples were evaluated using the paper disc agar diffusion method defined by National Committee for Clinical Laboratory Standard. The paper discs (6mm diameter) were incubated with the extracts overnight. After, the impregnated discs were placed on petri dishes inoculated with bacteria strains (10^5 CFU/mL). The petri dishes were incubated at 37 °C for 24 h. Finally, the diameters of the inhibition zones forming around the discs were measured to evaluate antibacterial activities of the extracts.

2.2.8. *In vivo toxicological analyses*

2.2.8.1. Fly rearing conditions of *Drosophila melanogaster* strains. The standard growth medium was prepared by dissolving sugar (43 g), agar (9 gr), semolina (90 gr), yeast (25 gr), antifungal drug (200 µl, Mikostatin-Deva Holding-228/97) and propionic acid (5 ml) in 500 ml of water (Chung *et al*., 2009; Yakovleva *et al*., 2016). 25 g of media was then proportioned into sterile glass cultures vials and the flies were kept in glass bottles at 22 °C.

2.2.8.2. The larval (eclosion) assay. The assay was performed according to Liu et al. with minor modifications (Liu *et al*., 2015). All of the *D. melanogaster* flies used in this study were Oregon R wild-type strains. 25 adult male and female *D. melanogaster* flies were placed into cultures bottles. After 48 ± 4 hours of incubation, 1st instar larvae were collected and rinsed with distilled water. Plant extracts (25 mg/L) and H_2O_2 (6.5 µg/mL) were directly applied to the growth media. The negative control was prepared without any treatment and the positive control was prepared by adding H_2O_2 (6.5 µg/mL). Equal numbers of 1st instar larvae were added into the experimental bottles and then incubated at 22 °C until they became adults. The pupae and eclosed adult fly numbers were counted (Liu *et al*., 2015). The puparation %, survival rate % and eclosion % were calculated according to the previous studies *(Depetris-Chauvin et al*., 2017; Liu *et al*., 2015; Macedo *et al*., 2017; Rand *et al*., 2014; Riaz *et al*., 2018) using the following formulas;

$$Puparation\ \% = \frac{Numberofpupae}{Numberoflarvae}\ x\ 100$$

$$Survival\ rate\ (\%) = \frac{Numberofadultflies}{Numberoflarvae}\ x\ 100$$

$$Eclosion\ \% = \frac{Numberofadultflies}{Numberofpupae}\ x\ 100$$

2.2.9. *Statistical analysis*

The standard error (SE) was calculated using three biological repeats, paired student *t* test was used and $p<0.05$ was determined as statistically significant for *in vivo* toxicological analyses. Other findings were presented as mean ± standard deviation ($\bar{X} \pm s$) of three biological repeats by Anova Test. All of the calculations and statistics of this study were performed by Microsoft Office Excel.

3. RESULTS

3.1. Enzyme Activity and Inhibition Results

The kinetic constants of the AChE enzyme obtained from *D. melanogaster* were presented in Table 1. They were calculated from Lineweaver-Burk equation using the acetylcholine substrate. The Michaelis constant (Km) and maximum reaction velocity (Vmax) values were calculated from the Lineweaver–Burk double reciprocal plots and values for the acetylcholine substrate were calculated as 1.94 mM and 17.95 EU/mL min, respectively.

Table 1. Kinetic values of AChE of *Drosophila melanogaster.*

Substrate	Km (mM)	Vmax (EU/mL min)	Vmax/Km (EU/mL min mM)
Acetycholine iodide	1.94	17.95	9.26

It is well known that most of the medicinal plants possess antioxidant activities. This property makes them very effective protectors against various diseases and memory deficits, in addition to their capacity of reducing the toxicities of toxic agents or other drugs (Karimi *et al*.,

2015). *RO*, *PT* and *SD* are the plants used to treat many diseases by local people. However, there was not enough data in the literature about their inhibition capacities on AChE that would make them natural alternatives of synthetic drugs without detrimental side effects leading to serious disorders in human metabolism (Colovic *et al.,* 2013). Therefore, this study aimed to analyse the AChE inhibition types and capacities of *RO, PT* and *SD* extracts prepared with EtOH, MeOH and water. The enzyme inhibition assay results were given in Table 2, Table 3 and at Figure 1.

Table 2. IC_{50} values of the plant samples on AChE of *Drosophila melanogaster.*

Samples	IC_{50} (µg/µL)		
	MeOH extract	EtOH extract	Aqueous extract
PT	0.86±0.05	2.54±0.11	4.76±0.24
SD	2.19±0.15	2.01±0.08	2.54±0.14
RO	1.21±0.07	0.57±0.02	1.98±0.13

Galanthamine (reference) 0.09 µg/µL.

According to Table 2,it was determined that extracts of *RO* had highest inhibitory activity among all of the plants, and its EtOH-extract showed the best inhibition activity with an IC_{50} value of 0.57±0.02 µg/µL, followed by the MeOH and water extracts with IC_{50} values of 1.21±0.07 and 1.98±0.13 µg/µL, respectively. Previously, Ozarowski et al. reported a study with *RO* L. leaf extract against AChE activity (Ozarowski *et al.,* 2013). They found that leaf extract prepared with 50% EtOH showed long-term inhibitory effect on AChE in rat's brain and they suggested that the *RO* leaf may be a possible option to prevent some neurodegenerative diseases (Ozarowski *et al.,* 2013). Our results were consistent with those findings. However, in another study, Orhan et al. reported that different extracts of *RO* prepared with methanol, petroleum ether, chloroform and ethyl acetate solvents were ineffective on AChE activity at 0.2 and 0.5 µg/µL concentrations (Orhan *et al*., 2008). However, in this study 25 µg/µL of *RO* extracts were used and strong inhibition was observed, so the difference between our results and Orhan *et al*. (Orhan *et al.,* 2008) may be occurred due to the differences of the doses.

According to the results given in Table 2, the extracts of *PT* also exhibited an effective inhibitory potential against AChE. The MeOH-extracts of *PT* exhibited the best inhibition potential (IC_{50}= 0.86±0.05µg/µL), followed by EtOH-extract (IC_{50}= 2.54±0.11µg/µL) and water extract (IC_{50}= 4.76±0.24µg/µL). The information in the literature is limited to compare with our data, but there is a study about the effect of *PT* extracts prepared with ethyl acetate and methanol on AChE activity (Orhan Erdogan *et al*., 2012). Researchers found that the *PT* extracts (25, 50, 100, and 200 µg/mL) did not show inhibitions against AChE but they selectively inhibited butyrylcholinesterase (BChE) activity at the tested concentrations (Orhan Erdogan *et al.*, 2012). In fact, it is an expected result to have different inhibition potentials at lower concentrations.

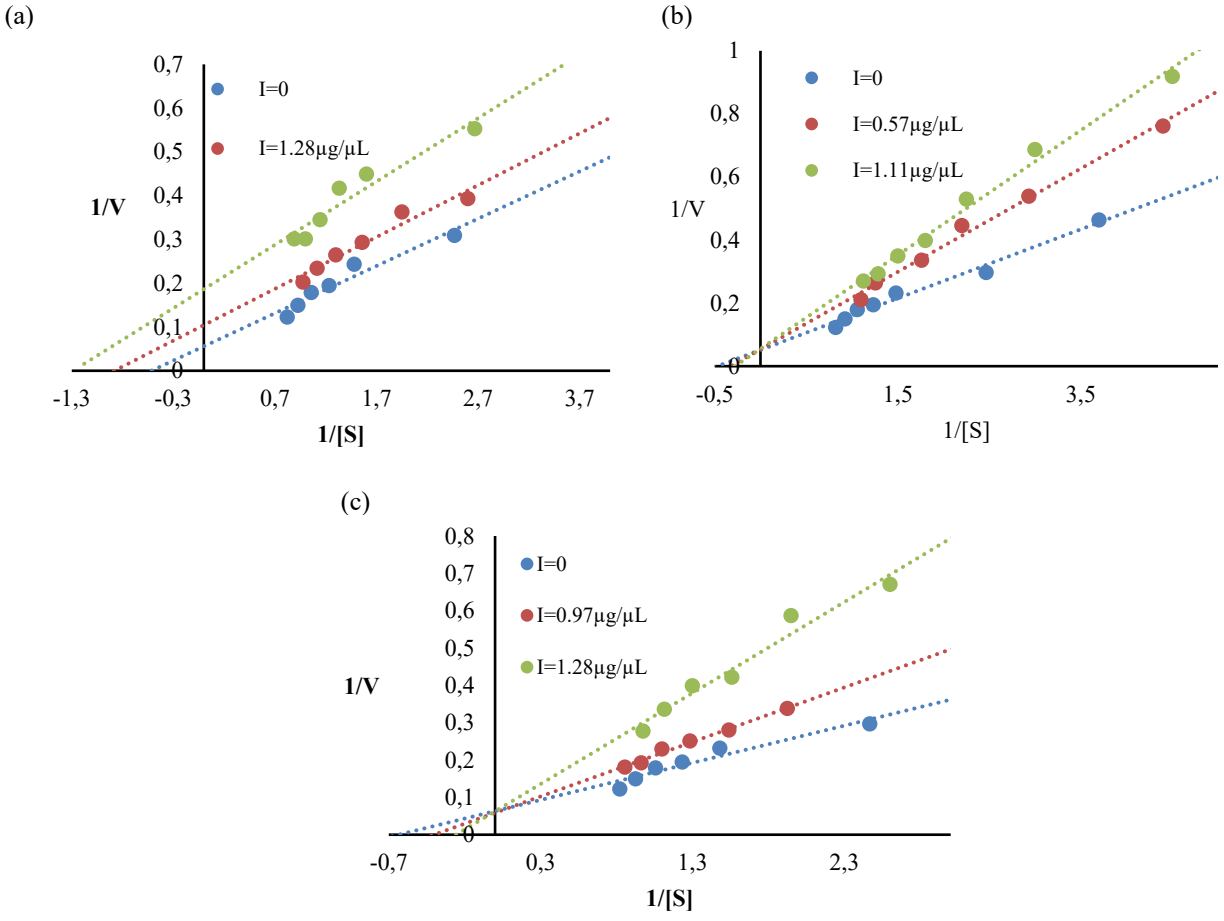
Table 3. Inhibition types and Ki values of *Drosophila melanogaster* AChE.

Inhibitors	I (µg/µL)	Ki (µg/µL)	Ki' (µg/µL)	Type of inhibition
RO (EtOH-extract)	1.28 1.88	--- ---	1.47 0.80	Uncompetitive
SD (EtOH-extract)	0.57 1.11	1.14 1.37	--- ---	Competitive
PT (MeOH-extract)	0.97 1.28	2.07 0.89	--- ---	Competitive

SD is a herbal tea consumed by local people because of its anti-inflammatory, antirheumatic, digestive and antimicrobial activities (Wang *et al*., 2006; Bahadori *et al*., 2016). In our study, all of the extracts of *SD* effectively reduced the AChE activity. EtOH-extract of *SD* showed the best inhibition activity against to AChE enzyme, and its IC_{50} value was found as 2.01±0.08µg/µL. IC_{50} values of MeOH and water extracts of *SD* were also determined as 2.19±0.25 and 2.54±0.14µg/µL, respectively. However, there isn't any research in the literature about the effects of *SD* on cholinergic system enzymes.

In addition, one of the results that make our study different from the literature is the determination of the inhibition types seen in Figure 1.Lower IC_{50} values result from the higher inhibition of AChE. Therefore, extracts with the lowest IC_{50} values were used to determine the inhibition types of each plant sample. Figure 1 shows the effects of *PT*-MeOH, *RO*-EtOH, and *SD*-EtOH inhibitors on *D. melanogaster* AChE using ACh as substrates. According to the results given in Figure 1(a), inhibition type of *RO* was determined as uncompetitive. Uncompetitive inhibition occurs when an inhibitor binds only to the complex formed between the enzyme and the substrate (ES complex). On the other hand, the competitive inhibitions were observed in the reactions between the *PT* and *SD* inhibitors and substrate catalysed by AChE enzyme (Figure 1(b), (c)). Moreover, Ki and Ki' values for inhibitors used are shown in Table 3. The inhibition constants given for the plant extracts (inhibitors) were obtained by fitting the experimental data with Lineweaver–Burk equation for competitive and uncompetitive inhibition. As a result, from Ki values in Table 3, it can be said that *RO* is a more effective inhibitor among the others due to lower Ki values. *SD* and *PT*, respectively, follow the inhibition efficiency.

Figure 1. Inhibition types of *RO*-EtOH (a), *SD*-EtOH (b) and *PT*-MeOH (c) extracts on AChE enzyme of *Drosophila melanogaster.*



3.2. Antioxidant capacity test results

It is known that oxidative stress has important roles both in early stages and the development of AD by activating multiple cell signalling pathways that contribute dangerous lesions (Feng & Wang, 2012). Thus, antioxidant therapies are considered an alternative or supplementary therapy option for AD (Feng & Wang, 2012). In fact, a great number of studies have examined the positive benefits of antioxidants to reduce or block neuronal death occurring in the pathophysiology of neurodegeneretive disorders like AD (Ramassamy, 2006).

In this study, DPPH and ABTS methods were used to determine the antioxidant properties of the plant extracts. According to the results, all extracts of *RO* and *SD* showed DPPH and ABTS radical scavenging activities equal or more than 90% (Table 4). Thus, it was obvious that the solvent types studied did not affect their antioxidant properties so their AChE inhibition capacities were not only dependent on their great antioxidant properties but also dependent on some unknown enzyme-specific inhibition mechanisms. Although DPPH and ABTS radical scavenging activities in EtOH-extract of *PT* were less than 90% (Table 4), they were quite high in MeOH-extract of *PT* (96.94±1.84 and 98.01±2.10%, respectively). These results were consistent with our findings showing that AChE inhibition capacity (Table 2) and natural anti-cholinesterase compositions (Table 5) were high in MeOH-extract of *PT*. In literature, researchers also have mentioned that *PT* extracts might provide neuro-protection to some extent with their strong antioxidant effects by metal-chelation (Orhan *et al*., 2012).

Table 4. Antioxidant scavenging activity and total phenolic content of the extracts.

	DPPH Scavenging Activity (%)			ABTS Scavenging Activity (%)			Total Phenolic Content (g/100g)		
Sample	MeOH extract	EtOH extract	Aqueous extract	MeOH extract	EtOH extract	Aqueous extract	MeOH extract	EtOH extract	Aqueous extract
PT	96.94±1.84	77.87±1.72	94.18±2.28	98.01±2.10	90.57±2.18	97.10±1.83	1.53±0.04	1.63±0.03	1.33±0.02
SD	93.47±2.37	94.41±1.51	90.83±1.53	98.18±2.36	97.41±2.26	96.58±1.86	1.74±0.04	1.31±0.03	2.05±0.05
RO	94.93±1.90	95.15±2.15	90.51±1.15	97.90±2.15	96.4±2.03	97.04±1.66	2.17±0.05	1.51±0.06	1.86±0.05

3.3. Total phenolic content results

It is well known that there is a linear correlation between total phenolic content values and antioxidant capacities (Johari & Khong, 2019). The total phenolic contents of all extracts of *RO*, *SD* and *PT* were determined in this study and expressed as g equivalent of gallic acid in 100g of extract. According to the findings given in Table 4, MeOH-extract of *RO* had the highest concentration of phenolic content (2.17±0.05 g/100g) among all extracts. It was followed by *SD* water extract as 2.05±0.05 g/100g and EtOH-extract of *PT* as 1.63±0.03 g/100g. The other extracts results were detected between 1.31±0.03 and 1.86±0.05 g/100g.

3.4. GC-MS results

Terpenes, the largest single class of compounds found in essential oils, have been shown to provide relevant protection under oxidative stress conditions like neurodegenerative disorders due to their antioxidant behaviors (Gonzalez-Burgos & Gomez-Serranillos, 2012). Therefore, to be able to identify the terpene compositions (monoterpenes, diterpenes and sesquiterpenes) of the plants that showed AChE inhibition, GC/MS analyses were performed. When the antioxidant effects were compared with respect to solvents (Table 4), it was clearly seen that MeOH-extracts of *PT* and *SD* showed the highest antioxidant capacities (above 95%) and the same extract of *RO* showed the highest phenolic content (2.17±0.05 g) which is also related to the antioxidant capacity. Therefore, MeOH-extracts were chosen in GC/MS analyses.

As seen in Table 5, twenty derivatives of terpenes were observed in *RO* extracts and among these compounds some monoterpenes like limonene (8.41%), borneol (7.49%), verbenone (6.19%) and camphor (4.68%) were at high concentrations. It was also clearly seen that acetylcholinesterase inhibitors such as 1,8-cineole, α-pinene, limonene, borneol, terpinene and verbenone, which are monoterpenes, were found in *RO* extracts. These results were consistent with the high AChE inhibition effects of *RO* extracts observed in this study.

Table 5. Composition of some terpene derivatives in *RO, PT* and *SD* extracts.

Compounds	*RO* (Area %)	*PT* (Area %)	*SD* (Area %)
1,8-cineole	0.77	0.19	1.60
α-pinen	0.01	0.09	0.001
Camphor	4.68	0.41	2.11
Borneol	7.49	0.51	1.19
Terpinene	0.12	0.28	0.09
α-terpineol	0.01	0.39	1.24
Verbenone	6.19	0.11	ND
Carvacrol	0.46	12.19	7.84
Viridiflorol	0.19	1.15	1.59
Caryophyllene	0.04	ND	0.33
Terpinolene	ND	0.43	0.67
Manool	ND	ND	1.72
Thymol	0.50	12.78	8.78
Limonene	8.41	0.01	ND
Linalool oxide	0.004	ND	ND
Linalool	0.013	0.03	0.02
Phytol	0.006	4.06	0.02
Dihydrocarveol	0.005	ND	ND
Totarol	1.61	ND	2.59

ND: non-detected

According to our GC/MS results with *PT* extracts (Table 5), thymol (12.78%) and carvacrol (12.19%) were found as main terpenes, followed by phytol (4.06%). It was also observed that *PT* extracts have many monoterpenes such as camphor, borneol, viridiflorol, α-terpineol, α-pinene and 1,8-cineole are natural anti-cholinesterase molecules (especially against to AChE) (Dave *et al*., 2000; Ozarowski *et al*., 2017). Thus, the GC/MS results of *PT* were consistent with our findings with AChE inhibition data.

GC/MS results showed that monoterpenes were predominant among numerous derivatives of terpenes in *SD* extracts. Among the compounds, thymol and carvacrol were found highest concentrations as 8.78 and 7.84%, respectively. Moreover, many monoterpenes such as totarol, camphor, manool, 1,8-cineole, borneol, α-terpineol and viridiflorol were determined in high concentration, too. The total concentration of terpene derivatives observed in *SD* extracts were approximately 30% of the extract and this can explain the strong inhibition activity of *SD* extracts observed in this study.

3.5. Antibacterial activity results

Antibacterial characteristic and the antioxidant activity of plant extracts are one of the most studied features for control of human and animal diseases of bacterial origin (Zhang *et al*., 2016). In addition, there is a hypothesis about the pathogenic bacteria invading the intestine can lead to brain dysfunctions by changing the flora of the intestine and AD may be associated with

that (Angelucci *et al.,* 2019). Therefore, the antibacterial effect of *RO, PT* and *SD* extracts were investigated in this study by disc diffusion method. According to the results given in Table 6 and Figure 2, all extracts showed antibacterial effects with obvious inhibition zones. However, the strongest antibacterial activity against *S. aureus* was found with the EtOH-extract of *RO* (25.77 mm inhibition zone) and the one against *E. coli* was found with the EtOH-extract of *SD* (19.52 mm inhibition zone). When the solvent types were compared, EtOH-extracts were found as more effective against *S. aureus* and *E. coli* than other extracts. Previous studies related to the different extracts of *RO, PT* and *SD* showed different antibacterial activity values (Bozin *et al*., 2007; Dhifi *et al*., 2012; Durak & Uçak, 2015; Fernández-López *et al*., 2005; Kilic *et al*., 2003). Compared with the literature values, the results of this study showed higher activities against *S. aureus* and *E. coli* than most of the others. All of the plants studied here can be regarded as natural antibiotics because they showed strong activities against both gram-positive and gram-negative bacteria. Therefore, *RO, PT* and *SD* should be considered as potential candidates for AD pharmaceutical applications with their important capacities to reduce AChE activity, their important phytochemical ingredients, and their prevention capacities from pathogenic bacteria.

Figure 2. Antibacterial activity results of the plants extracted in solvents against *S. aureus* and *E. Coli* performed by disc diffusion method (a- EtOH-extracts, c- MeOH-extracts, e- water-extracts against to *S. aureus* and b- EtOH-extracts, d- MeOH-extracts, f- Water-extracts against to *E. coli*) (C-Positive Control (Ampiciline), *RO-Rosmarinus officinalis*, *SD- Sideritis dichotoma*, *PT- Pistacia terebinthus*).

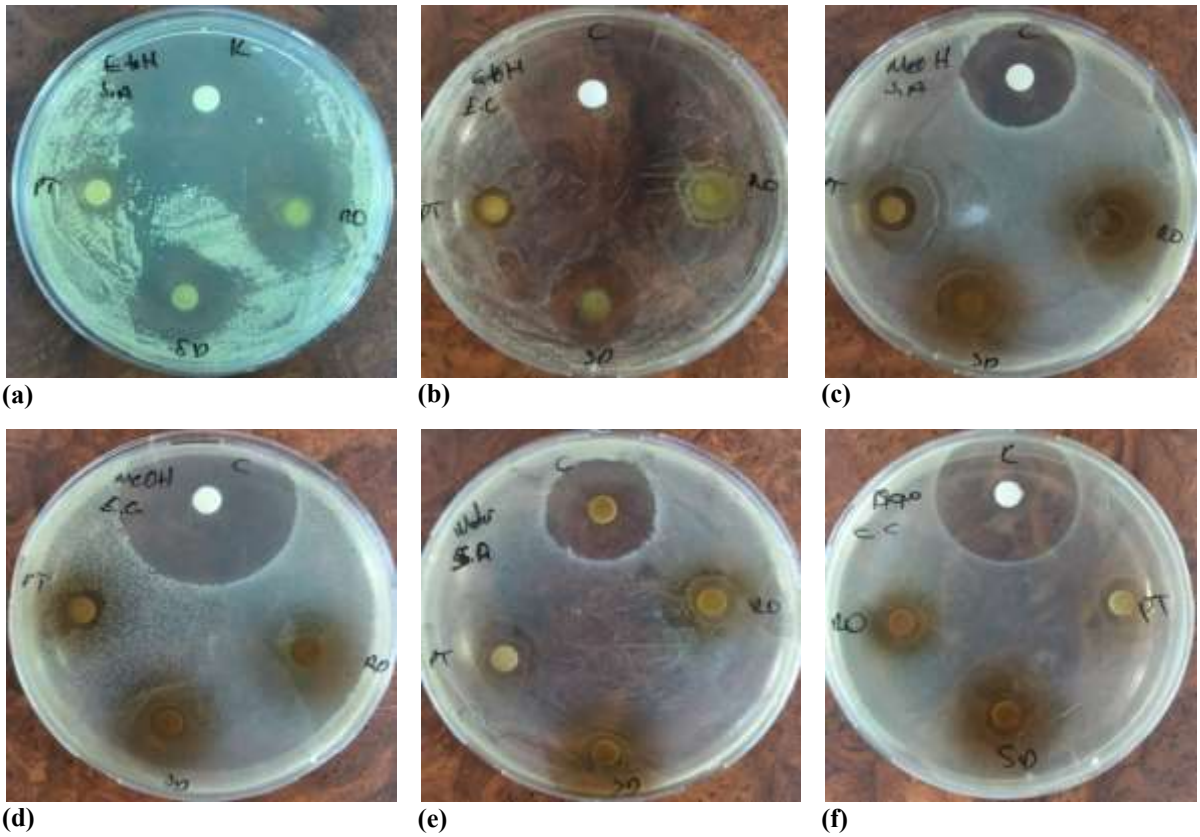


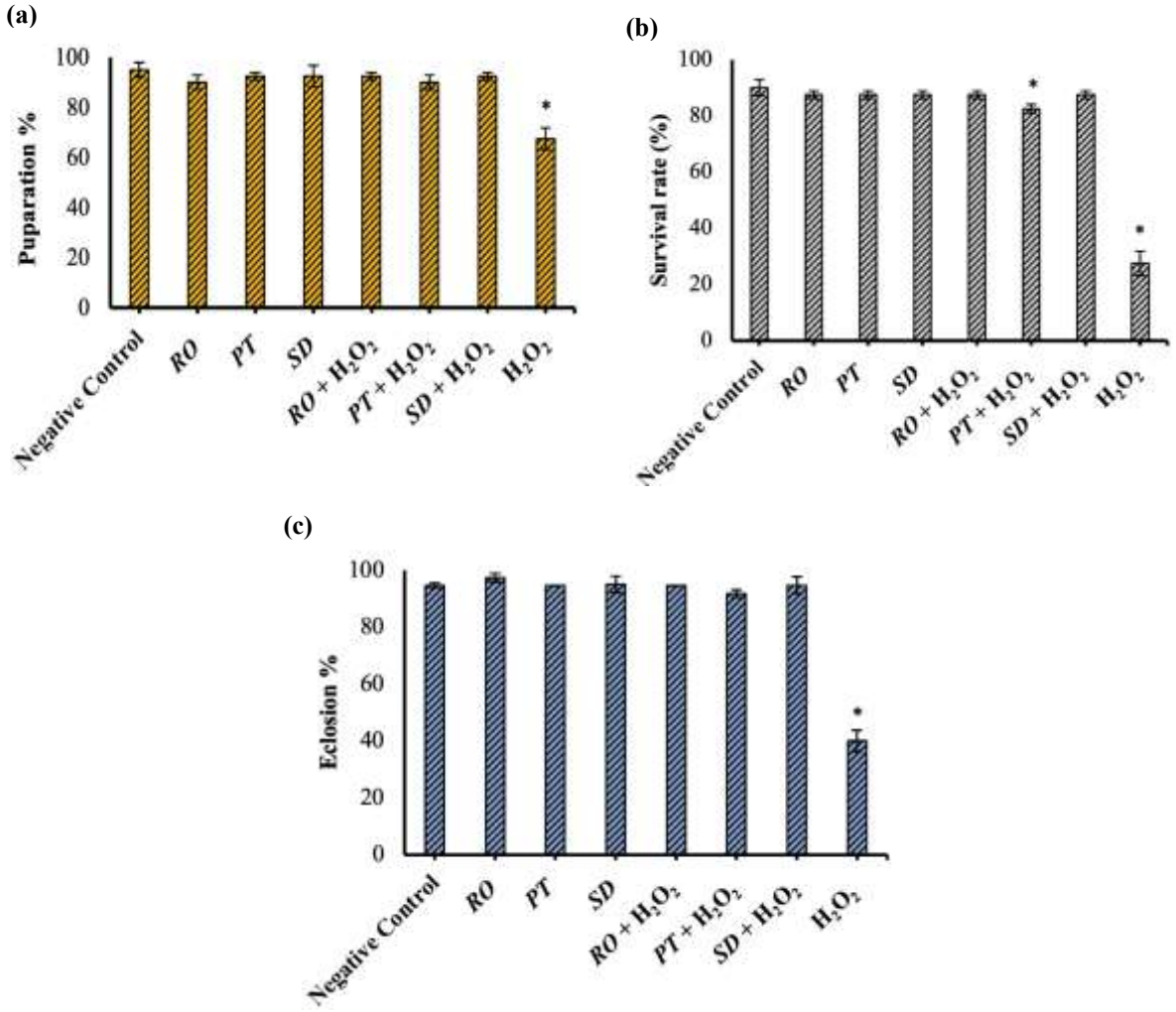
Table 6. Antibacterial activity results of the extracts against *S. aureus* and *E. coli* bacteria.

	Inhibition zone diameter (mm)					
	MeOH extract		EtOH extract		Aqueous extract	
Samples	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*
PT	11.54	10.32	9.07	11.22	10.38	9.03
SD	15.08	13.94	19.52	20.58	11.60	11.92
RO	12.04	15.03	12.69	25.77	12.14	13.84
Control (Ampicilin)	28.96	24.22	33.39	34.97	26.42	23.02

3.6. Results of *in vivo* toxicological analyses

The larval (eclosion) assays are recent techniques used to screen the effects of developmental susceptibility or tolerance to toxicants *in vivo* (Rand *et al.*, 2014). The assay is based on the relationship between toxic compounds and the metamorphosis process of *Drossophila* which is regulated by activation of four hormones (ecdysis triggering hormone, eclosion hormone, crustacean cardioactive peptid and bursicon) (Macedo *et al*., 2017). In order to determine the toxicologic effects of *RO, PT* and *SD* extracts *in vivo*, the larval (eclosion) assay was performed in this study and the results were given in Figure 3. The extracts that showed highest AChE inhibition values in this study (EtOH extract of *RO,* MeOH extract of *PT* and EtOH extract of *SD*) were analyzed in this assay. According to the results, it was clearly seen that H_2O_2 caused significant decreases ($p< 0.05$) in puparation %, survival rate % and eclosion % (Figure 3(a), (b), (c)). However, when the *RO, PT* and *SD* extracts were applied there was no significant change in puparation %, survival rate % or eclosion % (Figure 3(a), (b), (c)). In addition, extracts co-administered with H_2O_2 (*RO* + H_2O_2, *PT* + H_2O_2 and *SD* + H_2O_2) showed similar puparation and eclosion % values like negative control (Figure 3(a), (c)). Although there is a decrease in survival rate (%) of cultures treated with *PT* + H_2O_2, the value was significantly higher than the ones treated with H_2O_2 alone ($p<0.05$). Therefore, it can be concluded that none of the extracts used in this study was toxic for *Drossophila* and they ameliorated the H_2O_2 induced decrease of puparation %, survival rate % and eclosion % values. To date, no study has demonstrated the developmental susceptibility or tolerance to *RO, PT* and *SD* extracts *in vivo*. However, there are some studies in literature about *in vivo* toxicological effects of different plant species on *Drosophila (Liu et al*., 2015; Macedo *et al*., 2017; Riaz *et al*., 2018). For example, Liu et al. studied the effects of *Coriandrum sativum*, *Nardostachys jatamansi*, *Polygonum multiflorum*, *Rehmannia glutinosa* and *Sorbus commixta* on *Drosophila* strains and found significant increases in survival rate % with those plant extracts compared to the ones with AD phenotypes *(Liu et al*., 2015). In another study, it was investigated that hydroalcoholic extract from leaves of *Senecio brasilienis* (Spreng) Less. caused significant decrease in the eclosion rate of flies at higher concentrations (1mg/ml) (Macedo *et al*., 2017). The toxicity of petroleum extract of *Euphorbia prostrata*, *Parthenium hysterophorus*, *Fumaria indica, Chenopodium murale* and *Azadirachta indica* against *D. melanogaster* were also studied (Riaz *et al*., 2018). According to Riaz et al., *E. prostrata* was the only one with high mortality (51.64%) at 30% concentration and it was significantly higher than the negative control after 72 h of incubation.

Figure 3. EtOH extract of *R. officinalis* (*RO*), MeOH extract of *P. terebinthus* (*PT*) and EtOH extract of *S. dichotoma* (*SD*) extracts ameliorated the decreased **a-**puparation %, **b-**survivalrate (%) and **c-**eclosion % of *Drosophila.* * indicates that $p< 0.05$ compared to negative control.



4. DISCUSSION and CONCLUSION

Although the pathogenesis of AD has not been fully deciphered yet, increased activity of AChE and oxidative stress are considered the main reasons for (Cavdar *et al.,* 2019; Zhao & Zhao, 2013). Natural compounds have become an emerging and promising area of research for the therapy of neurodegenerative diseases like AD because of their strong antioxidant capacities (Ramassamy, 2006). Therefore, this study identified the inhibition capacities of *RO*, *PT* and *SD* extracts on AChE, the antioxidant properties, phenolic contents, terpene compositions, antibacterial effects, and *in vivo* toxicities of the plants. All of the plant extracts showed strong inhibitory effects on AChE activity. The inhibition type of *RO* was uncompetitive, while *SD* and *PT* extracts showed competitive inhibition on AChE activity. Moreover, GC/MS results showed that carvacrol and thymol were the major monoterpenes of *PT* and *SD* extracts, while limonene and borneol were the main monoterpenes of *RO* extracts. The strongest antibacterial activities were observed with EtOH extract of *RO* (25.77 mm) against *S. aureus* and with EtOH extract of *SD* (19.52 mm) against *E. coli*. To conclude, all of the plant extracts studied were capable of inhibiting the AChE activity and this observation was compatible with their important biochemical compositions revealed in this study. It was also determined that their great potential as antibacterial agents and non-toxic characteristics make them important

candidates for pharmaceutical applications like anticholinesterase drugs or starter compounds for synthesizing more effective AChE inhibitors.

Acknowledgments

The authors would like to thank the Balikesir University Science and Technology Application and Research Center for the Grant provided for this research and Prof. Dr. Serap Doğan for her assistance in plant identification.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship contribution statement

Mehmet Emin Diken: Investigation, Methodology, Supervision, Resources, and Writing - original draft. **Begumhan Yilmaz Kardas**: Investigation, Methodology, Resources, and Writing -original draft.

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