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The Effects of Inhaled *Pimpinella peregrina* Essential Oil on Scopolamine-Induced Memory Impairment, Anxiety, and Depression in Laboratory Rats

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Abstract In the present study, we identified the effects of inhaled *Pimpinella peregrina* essential oil (1 and 3 %, for 21 continuous days) on scopolamine-induced memory impairment, anxiety, and depression in laboratory rats. Y-maze and radial arm-maze tests were used for assessing memory processes. Also, the anxiety and depressive responses were studied by means of the elevated plus-maze and forced swimming tests. The scopolamine alone-treated rats exhibited the following: decrease of the spontaneous alternation percentage in Y-maze test, increase of the number of working and reference memory errors in radial arm-maze test, along with decrease of the exploratory activity, the percentage of the time spent and the number of entries in the open arm within elevated plus-maze test and decrease of swimming time and increase of immobility time within forced swimming test. Inhalation of the *P. peregrina* essential oil significantly improved memory formation and exhibited anxiolytic- and antidepressant-like effects in scopolamine-treated rats. Our results suggest that the *P. peregrina* essential oil inhalation ameliorates scopolamine-induced memory impairment, anxiety, and depression. Moreover, studies on the *P. peregrina* essential oil may open a new therapeutic window for the prevention of neurological abnormalities closely related to Alzheimer's disease.

Keywords *Pimpinella peregrina* essential oil · Scopolamine · Memory · Anxiety · Depression · Alzheimer's disease

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that leads to dementia and affects approximately 10 % of the population older than 65 years of age. In AD brains, severe neurodegenerative alterations occur. Loss of neurons and synapses, atrophy, and the selective depletion of neurotransmitter systems such as acetylcholine in the hippocampus and cerebral cortex are within these alterations [1].

The two pathological hallmarks of AD include senile plaques consisting beta-amyloid peptides and neurofibrillary tangles formed by hyperphosphorylation and abnormal deposition of *tau* proteins [2]. Multifactorial causes require multi-targeted treatment concepts [3]. AD is associated with an impairment of daily activities, behavior disturbances, and a variety of neuropsychiatric symptoms [4]. To diagnose AD in early stages is extremely important, so that there is increasing intention to identify a behavioral or physiological markers for AD. Although, cognitive impairment is generally believed to be a normal consequence of aging, there is evidence to suggest that cognitive decline is not solely due to aging alone, and can be used to identify people who are specifically at risk for developing dementia [5]. Memory deficits are observed even at the early stages of AD [6] and are associated with cholinergic deficits in hippocampus and cerebral cortex [7].

Neuropsychiatric symptoms such as depression, apathy, aggression, and psychosis are now recognized as core features of AD, and there is a general consensus that greater symptom

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severity is predictive of faster cognitive decline, loss of independence, and even shorter survival. Whether these symptoms result from the same pathogenic processes responsible for cognitive decline or have unique etiologies independent of AD-associated neurodegeneration is unclear [8].

Scopolamine is a nonselective muscarinic receptor antagonist and it consistently has produced emotional based learning impairment in rodents [9]. Scopolamine has been used as a standard/reference drug for inducing age- and dementia-related cognitive deficits in healthy humans and animals. The pharmacological model of "cholinergic amnesia" using scopolamine became popular after the cholinergic hypothesis was formed [10].

Pimpinella peregrina is a member of the Apiaceae family comprising approximately 150 species distributed in the northern hemisphere. *Pimpinella* species are very common in Turkey [11] and are represented by 23 species, including *P. peregrina* [12].

The seeds of *Pimpinella* species have been used in folk medicine, pharmacy and food industry, mainly due to its essential oil attributes. Essential oil forms the basis of all flavored drinks, such as pastis, ouzo, raki, arak, and many others [13]. Some of the members of *Pimpinella* genus were cultivated by Romans, Greeks, and Egyptians for their aromatic seeds used in medicine and as a condiment. Their fruits have been used for medicinal purposes and also in cooking, and are listed in British, German, and European Pharmacopoeia. These plants, and especially their seeds, have been used as appetizers, tranquillizers, and diuretic drugs in Turkish folk medicine [14].

Despite extensive knowledge about the effects of *Pimpinella* species extracts, there is no study, clarifying the possible cognitive-enhancing, anxiolytic and antidepressant activities of *P. peregrina* essential oil in scopolamine-induced a rat model of cholinergic amnesia.

In this study, we examined the therapeutic effects of *P. peregrina* essential oil on memory processes, anxiety, and depressive-like behaviors in scopolamine-treated rats. Correlation between the behavioral scores of scopolamine-treated rats, as a result of inhalation of essential oil was also investigated.

Materials and Methods

Plant Materials and Volatile Oil Preparation

Aerial parts of *P. peregrina* were collected in the flowering stage in Adiyaman, Eastern Anatolia, Turkey, in June 2013. The samples of the plants were identified by Prof. Dr. Eyup Bagci and a voucher specimen was registered and deposited in the Herbarium of Department of Biology, Firat University for ready reference. The oil was extracted by hydro-distillation for 3 h using a Clevenger-type apparatus. The total essential oil yield was 0.7 % (*v*/*w*, dry material).

Gas Chromatography (GC-MS/GC-FID) Analysis

GC-MS analysis of the *P. peregrina* essential oil was performed in Plant Products and Biotechnology Research Laboratory (BUBAL), Firat University, using Hewlett Packard-Agilent 5973N GC-MS system with 6890 GC equipped with a flame ionization detector (FID). HP-5 MS column (30 m × 0.25 mm i.d., film thickness (0.25 μm)) was used with helium as the carrier gas. Injector temperature was 250 °C, and split flow was 1 ml/min. The GC oven temperature was kept at 70 °C for 2 min and programmed to 150 °C at a rate of 10 °C/min and then kept constant at 150 °C for 15 min to 240 °C at a rate of 5 °C/min. Alkanes were used as reference points in the calculation of retention indices (RI). MS were taken at 70 eV and a mass range of 35–425. The identification of the compounds was based on comparison of their retention indices (RI), their retention times (RT) and mass spectra with those obtained from authentic Wiley libraries (available through Hewlett Packard) and the literature [15].

Animals

Thirty-six male Wistar rats weighing 250 ± 50 g at the start of the experiment were used. The animals were housed in a temperature and light-controlled room (22 °C, a 12-h cycle starting at 08:00 h) and were fed and allowed to drink water ad libitum. The rats were divided into six groups (six animals per group): (1) control group received 0.9 % saline with 1 % Tween 80 treatment; (2) scopolamine (Sco) alone-treated group received 0.9 % saline with 1 % Tween 80 treatment, as negative control; (3) diazepam alone-treated group (DZP, 1.5 mg/kg) received 0.9 % saline with 1 % Tween 80 treatment, as positive control; (4) tramadol alone-treated group (TRM, 10 mg/kg) received 0.9 % saline with 1 % Tween 80 treatment, as positive control; (5) scopolamine-treated group received by inhalation *P. peregrina* essential oil 1 % (Sco + PIM1%); and (6) scopolamine-treated group received by inhalation *P. peregrina* essential oil 3 % (Sco + PIM3%). Diazepam and tramadol alone-treated groups were used as positive controls within elevated plus-maze and forced swimming tests. Control, DZP, TRM-, and scopolamine alone-treated groups were caged in the same conditions but in the absence of the tested essential oil. They were subjected to inhale 0.9 % saline with 1 % Tween 80 solution.

Inhalation Apparatus and Drug Administration

The inhalation apparatus consisted of a Plexiglas chamber (50 × 40 × 28 cm). Two chambers were used, one for the control and scopolamine alone-treated animals, which were

exposed to 0.9 % saline with 1 % Tween 80 solution and the other one for the experimental animals, which were exposed to *P. peregrina* essential oil. *P. peregrina* essential oil was diluted with 1 % Tween 80 (*v*/*v*). *P. peregrina* essential oil exposure (200 μl, either 1 or 3 %) was via an electronic vaporizer (Oregon Scientific WS113) placed at the bottom of chamber, but out of reach of the animals. Regarding concentrations to be used in the pharmacological tests, we selected 1 % essential oil normally used in aromatherapy and a higher concentration (3 %) in order to emphasize the effects [16]. Rats in the *P. peregrina* essential oil groups were exposed to oil vapors for controlled 15 min period, daily, for 21 continuous days. Chambers were always cleaned up (10 % ethanol solution). Scopolamine hydrobromide (Sigma-Aldrich, Germany) was used as negative control and was dissolved in an isotonic solution (0.9 % NaCl) and 0.7 mg/kg scopolamine was injected intraperitoneally (i.p.), 30 min before the behavioral testing. Diazepam (1.5 mg/kg, Sigma-Aldrich, Germany) and tramadol hydrochloride (10 mg/kg, Sigma-Aldrich, Germany) were used as positive controls and were injected intraperitoneally (i.p.) in a volume of 1 ml/kg in laboratory rats, 1 h before behaviorally tested in the elevated plus-maze and forces swimming tests. Scopolamine was given once to all scopolamine groups in the Y-maze task and for 7 consecutive days to all scopolamine groups before the radial arm-maze task. An experimental schedule is indicated in Fig. 1.

Y-Maze Test

Short-term memory was assessed by spontaneous alternation behavior in the Y-maze task. The Y-maze used in the present study consisted of three arms (35 cm long, 25 cm high and 10 cm wide) and an equilateral triangular central area. Fifteen minutes after the inhalation of *P. peregrina* essential oil (PIM1% and PIM3%), rats were placed at the end of one arm and allowed to move freely through the maze for 8 min. An arm entry was counted when the hind paws of the rat were completely within the arm. Spontaneous alternation behavior was defined as entry into all three arms on consecutive choices. The number of maximum spontaneous alternation behaviors was then the total number of arms entered minus 2 and percent spontaneous alternation was calculated as (actual alternations/maximum alternations) × 100 [17]. Spontaneous alternation behavior is considered to reflect spatial working memory, which is a form of short-term memory. The maze was cleaned with a 10 % ethanol solution and dried with a cloth before the next animal was tested.

Radial Arm-Maze Test

The radial arm-maze used in the present study consisted of eight arms, numbered from 1 to 8 (48 cm × 12 cm), extending radially from a central area (32 cm in diameter). The apparatus was placed 50 cm above the floor and surrounded by various extra-maze visual cues placed at the same position during the study. At the end of each arm there was a food cup that had a single 50 mg food pellet. Prior to the performance of the maze task, the animals were kept on restricted diet and body weight was maintained at 85 % of their free-feeding weight over a week period, with water being available ad libitum. Before the actual training began, three or four rats were simultaneously placed in the radial arm-maze and allowed to explore for 5 min and take the food freely. The food was initially available throughout the maze, but was gradually restricted to the food cup. The animals were trained for 4 days to run to the end of the arms and consume the bait. To evaluate the basal activity of rats in radial arm-maze, the rats were given 5 consecutive training trials per day to run to the end of the arms and consume the bait. The training trial continued until all 5 baits have been consumed or until the 5 min have elapsed which have been set as the performance criteria. After adaptation, all rats were trained with 1 trial per day. Briefly, 15 min after the inhalation of *P. peregrina* essential oil (PIM1% and PIM3%), each animal was placed individually in the center of the maze and subjected to working and reference memory tasks, in which same 5 arms (nos. 1, 2, 4, 5, and 7), were baited for each daily training trial. The other 3 arms (nos. 3, 6 and 8) were never baited. The selection of the baited arms is based on the fact that animals prefer to solve the maze using an adjacent arm selection strategy. In this case, we altered adjacent arm patterning behavior by only baiting 5 arms (nos. 1, 2, 4, 5, and 7) subjecting animals to change their strategy and avoid the unbaited arms. An arm entry was counted when all four limbs of the rat were within an arm. Measures were made of the

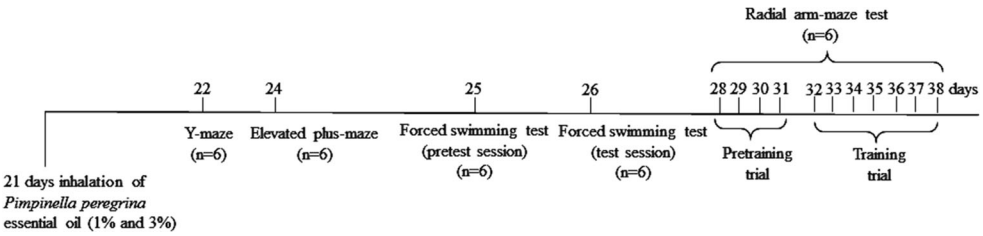

Fig. 1 Schedule of the experiment. Rats in the *P. peregrina* essential oil groups were exposed to oil vapors for controlled 15 min period, daily, for 21 continuous days. Behavior tests were performed immediately next day after the last exposure to oil vapors

number of working memory errors (entering an arm containing food, but previously entered) and reference memory errors (entering an arm that was not baited) [17]. Reference memory is regarded as a long-term memory for information that remains constant over repeated trials (memory for the positions of baited arms), whereas working memory is considered a short-term memory in which the information to be remembered changes in every trial (memory for the positions of arms that had already been visited in each trial). The maze was cleaned with a 10 % ethanol solution and dried with a cloth before the next animal was tested.

Elevated Plus-Maze Test

Behavior in elevated plus-maze test (EPM) is also utilized to assess exploration, anxiety, and motor behavior. The EPM consists of four arms, 49 cm long and 10 cm wide, elevated 50 cm above the ground. Two arms were enclosed by walls 30 cm high and the other two arms were exposed. 15 min after the inhalation of *P. peregrina* essential oil (PIM1% and PIM3%), each rat was placed in the center of the maze facing one closed arm. Behavior was observed for 5 min, and the time spent and number of entries into the open and enclosed arms was counted [18]. The percentages of time spent in the open arms (time spent in the open arms/time spent in all arms × 100) were calculated. In addition, the total number of open- and enclosed-arm entries (number of crossing), which indicates the exploratory activity of animals [19], was measured. An entry was defined as an animal placing all four paws into an arm, and no time was recorded when the animal was in the central area. The maze floor was cleaned with cotton and 10 % ethanol solution between subjects.

Forced Swimming Test

The forced swimming test (FST) is the most widely used model for assessing depressive-like response [20]. The depressive-like response was assessed, basically using the same method described by Campos et al. [21], but with modification. On the first day of the experiments (pretest session), rats were individually placed into cylindrical recipients (diameter 30 cm, height 59 cm) containing 25 cm of water at 26 ± 1 °C. The animals were left to swim for 15 min before being removed, dried, and returned to their cages. The procedure was repeated 24 h later, in a 6-min swim session (test session), 15 min after the inhalation of *P. peregrina* essential oil (PIM1% and PIM3%). During the test session, the following behavioral responses were recorded: (1) immobility (time spent floating with the minimal movements to keep the head above the water) and (2) swimming (time spent with active swimming movements).

Statistical Analysis

Behavioral scores within Y-maze, radial arm-maze, elevated plus-maze, and forced swimming tests were analyzed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test using GraphPad Prism 6 software for Windows, La Jolla, California, USA. In order to evaluate differences between groups in the radial arm-maze task, separate repeated-measures ANOVA was calculated on the number of working memory errors and the number of reference memory errors with group (Control, Sco, Sco + PIM1% and Sco + PIM3%) as between-subject factor and days (1 to 7) as within-subjects factors. All results are expressed as mean ± standard error of mean (SEM). F values for which $p < 0.05$ were regarded as statistically significant. Pearson's correlation coefficient and regression analysis were used in order to evaluate the connection between behavioral measures.

Results

Chemical Composition of the *P. peregrina* Essential Oil

The GC-MS/GC-FID analysis of the volatile profiles in *P. peregrina* essential oil is listed in Table 1. A total of 20 different compounds were isolated which constituted 90.1 % (*w*/*w*) of the total essential oil. As a result of GC-MC analysis, *trans*-pinocarveol (35.1 %) was the major compound for the sample studied. Additionally, other major compounds were pregeijerene (15.1 %), α-cubebene (12.4 %), (+)-epi-bicyclosesquiphellandrene (7.5 %), α-terpineol (6.7 %), allocymene (4.0 %), iso-spathulenol (3.1 %), and bicyclogermacrene (1.8 %). Monoterpenes represented 70 % of the essential oil and consisted of sabinene (0.3 %), DL-limonene (0.1 %), β-ocimene (0.2 %), γ-terpinene (0.1 %), linalool (0.1 %), allocymene (4.0 %), *trans*-pinocarveol (35.1 %), 3-cyclohexen-1-ol (0.6 %), α-terpineol (6.7 %), thymol (0.2 %), pregeijerene (15.1 %), and (+)-epi-bicyclosesquiphellandrene (7.5 %). Sesquiterpenes represented 20 % of the essential oil and consisted of α-cubebene (12.4 %), β-caryophyllene (0.9 %), germacrene D (0.3 %), bicyclogermacrene (0.4 %), spathulenol (1.8 %), caryophyllene oxide (1.1 %), and iso-spathulenol (3.1 %), followed by *p*-cresol (0.1 %), a methylphenol compound.

Effect of the *P. peregrina* Essential Oil on Spatial Memory in Y-Maze Test

Analyses of the spontaneous alternation percentage within Y-maze task showed significant overall differences between all groups ($F(3,20) = 7.30$), $p < 0.001$ (Fig. 2b).

Table 1 Chemical composition (%) of identified compounds in the essential oil of *Pimpinella peregrina* aerial parts

No.	Compounds	RI	Percentage
1	Sabinene	1051	0.3
2	DL-limonene	1096	0.1
3	β-Ocimene	1107	0.2
4	γ-Terpinene	1115	0.1
5	*p*-Cresol	1128	0.1
6	Linalool	1146	0.1
7	Allocymene	1170	4
8	*trans*-Pinocarveol	1176	35.1
9	3-Cyclohexen-1-ol	1203	0.6
10	α-Terpineol	1215	6.7
11	Thymol	1274	0.2
12	Pregeijerene	1290	15.1
13	α-Cubebene	1330	12.4
14	β-Caryophyllene	1408	0.9
15	(+)-Epi-bicyclosesquiphellandrene	1403	7.5
16	Germacrene D	1432	0.3
17	Bicyclogermacrene	1442	0.4
18	Spathulenol	1493	1.8
19	Caryophyllene oxide	1496	1.1
20	Iso-spathulenol	1604	3.1
Total			90.1

RI: experimental retention indices relative to *n*-alkanes on the HP-5 MS column

Both doses of inhaled *P. peregrina* essential oil, but especially 3 %, significantly improved memory formation in scopolamine-treated rats as compared to scopolamine alone-treated rats. The changes in the spontaneous alternation percentages of scopolamine-treated rats exposed to *P. peregrina* essential oil are not related to the changes in motor activity, as evidenced by the number of arm entries as compared to scopolamine alone-treated rats (Fig. 2a).

Effect of the *P. peregrina* Essential Oil on Spatial Memory in Radial Arm-Maze Task

To investigate whether exposure to *P. peregrina* essential oil of scopolamine-treated rats affects spatial memory, the rats were further evaluated in the radial arm-maze task.

For working memory errors, repeated-measures ANOVA revealed a significant time difference ($F(6,140) = 2.97$, $p < 0.001$) and a significant group difference ($F(3,140) = 6.41$, $p < 0.0001$) (Fig. 3a).

For reference memory errors, repeated-measures ANOVA revealed a significant group difference ($F(3,140) = 15.86$, $p < 0.0001$) (Fig. 3b).

Effect of the *P. peregrina* Essential Oil on Elevated Plus-Maze Behavior

In the elevated plus-maze task ANOVA revealed a significant overall differences between all groups ($F(4,25) = 59.18$, $p < 0.0001$) on the percentage of the time spent in the open arms (Fig. 4a). Both doses of the *P. peregrina* essential oil, but especially 1 %, significantly increased the percentage of the time spent in the open arms in scopolamine-treated rats as compared to scopolamine alone-treated rats.

Also, ANOVA revealed a significant overall differences between all groups ($F(4,25) = 7.38$, $p < 0.0001$) on the number of open-arm entries (Fig. 4b). Inhalation of the *P. peregrina* essential oil, but especially 1 %, significantly increased on the number of open-arm entries of scopolamine-treated rats as compared to scopolamine alone-treated group.

ANOVA revealed a significant overall differences between all groups ($F(4,25) = 13.62$, $p < 0.0001$) on the number of crossing (exploratory activity) (Fig. 4c). Inhalation of the *P. peregrina* essential oil, but especially 1 %, significantly increased the number of crossing of scopolamine-treated rats as compared to scopolamine alone-treated rats.

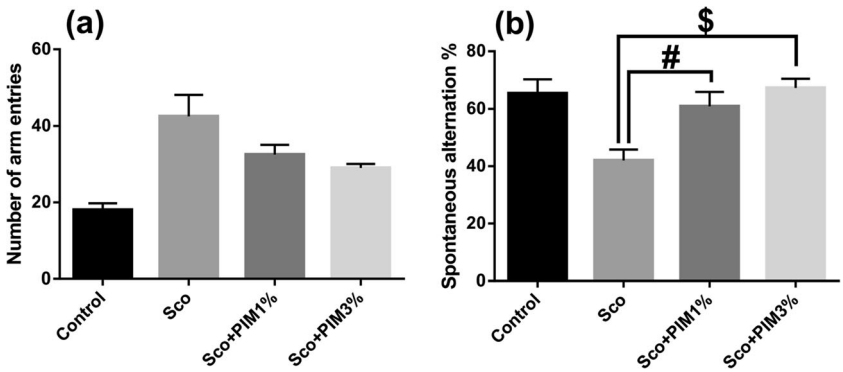

Fig. 2 Effects of the inhaled *P. peregrina* essential oil (PIM1% and PIM3%) in the Y-maze on the number of arm entries (**a**) and spontaneous alternation % (**b**) in the scopolamine (Sco)-treated rats. Values are means ± SEM ($n = 6$ animals per group). For Turkey's post hoc analyses —$^{\#}$Sco vs. Sco + PIM1% $p < 0.01$ and $^{\$}$Sco vs. Sco + PIM3% $p < 0.001$

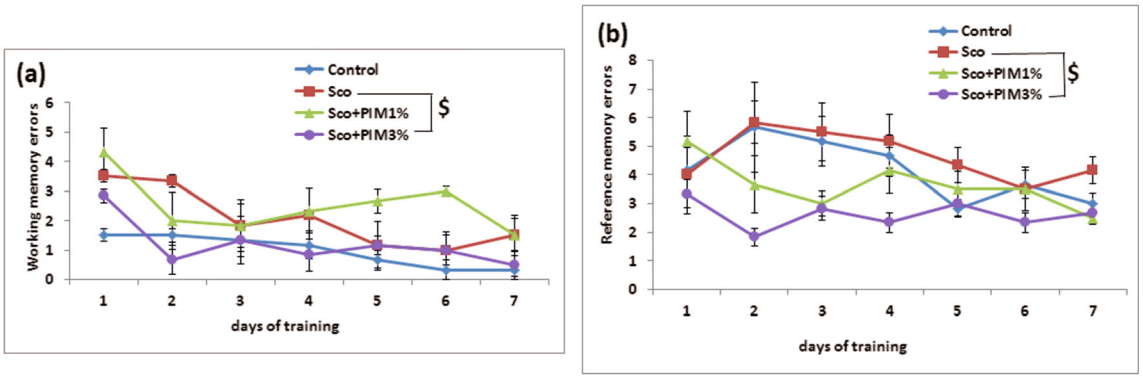

Fig. 3 Effects of the inhaled *P. peregrina* essential oil (PIM1% and PIM3%) on the working memory errors (**a**) and the reference memory errors (**b**) during 7 days training in the radial arm-maze in the scopolamine (Sco)-treated rats. Values are means ± SEM ($n = 6$ animals per group). For Turkey's post hoc analyses—$^{\$}$Sco vs. Sco + PIM3% $p < 0.01$ (**a**) and $^{\$}$Sco vs. Sco + PIM3% $p < 0.001$ (**b**)

The diazepam treatment, as positive control, significantly increased the percentage of the time spent in the open arms, the number of open-arm entries, and the number of crossing, acting as anxiolityc agent.

Effect of the *P. peregrina* Essential Oil in the Rat Forced Swimming Test

In the forced swimming test, ANOVA revealed a significant overall differences between all groups on the swimming time ($F(4,25) = 31.33$, $p < 0.0001$) (Fig. 5a) and on the immobility time ($F(4,25) = 28.43$, $p < 0.0001$) (Fig. 5b). Both doses of *P. peregrina* essential oil, but especially 1 %, significantly increased swimming time and decreased immobility time of scopolamine-treated rats as compared to scopolamine alone-treated rats.

The tramadol treatment, as positive control, increased the swimming time and decreased the immobility time, acting as antidepressant agent.

More importantly, when linear regression was determined, significant correlations between the percentage of the spontaneous alternation vs. the number of the open arm entries ($n = 24$, $r = 0.503$, $p < 0.01$) (Fig. 6a), the percentage of the spontaneous alternation vs. swimming time ($n = 24$,

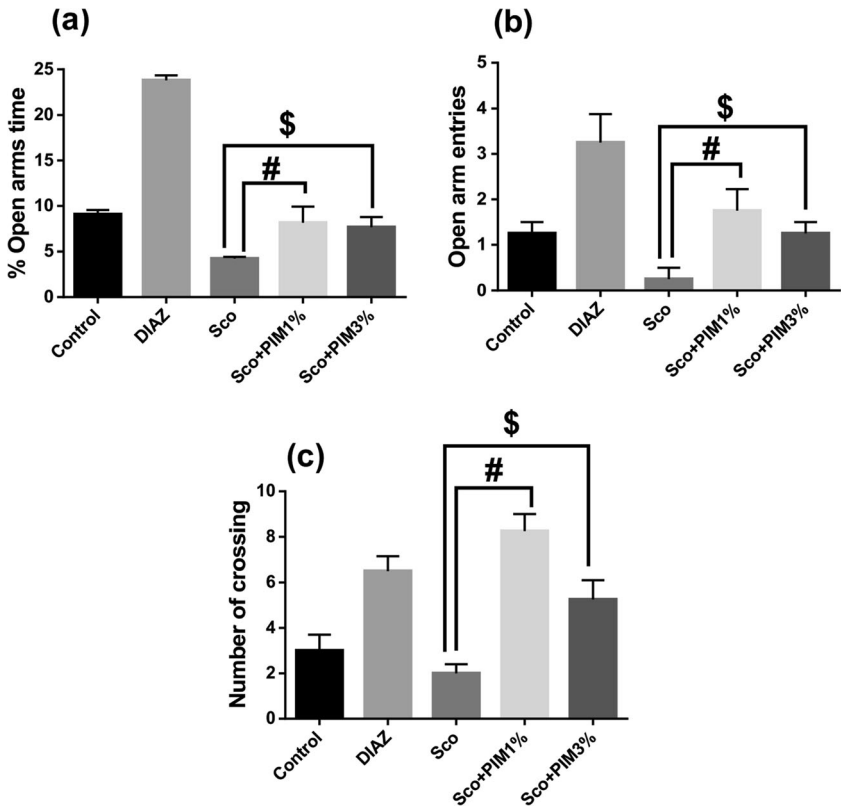

Fig. 4 Effects of the inhaled *P. peregrina* essential oil (PIM1% and PIM3%) in the elevated plus-maze test on the percentage of the time spent in the open arms (**a**), the number of open-arm entries (**b**), and number of crossing (**c**) in the scopolamine (Sco)-treated rats. Values are means ± SEM ($n = 6$ animals per group). For Turkey's post hoc analyses—$^{\#}$Sco vs. Sco + PIM1% $p < 0.01$ and $^{\$}$Sco vs. Sco + PIM3% $p < 0.05$ (**a**), $^{\#}$Sco vs. Sco + PIM1% $p < 0.001$ and $^{\$}$Sco vs. Sco + PIM3% $p < 0.01$ (**b**) and $^{\#}$Sco vs. Sco + PIM1% $p < 0.0001$ and $^{\$}$Sco vs. Sco + PIM3% $p < 0.01$ (**c**)

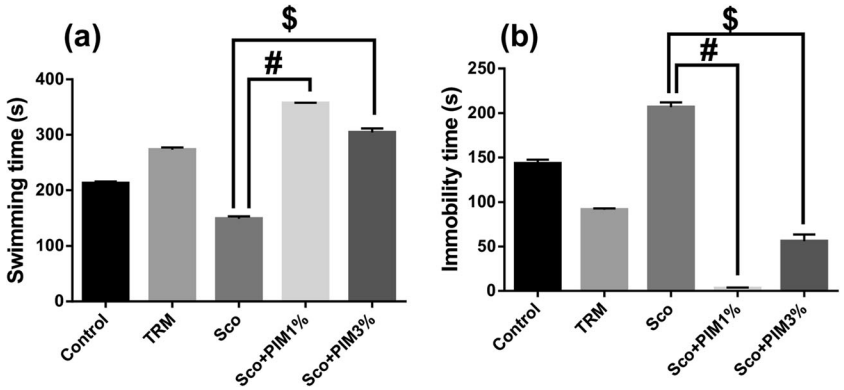

Fig. 5 Effects of the inhaled *P. peregrina* essential oil (PIM1% and PIM3%) on swimming time (**a**) and immobility time (**b**) in the scopolamine (Sco)-treated rats during the 6-min period in the forced swimming test. Values are means ± SEM ($n = 6$ animals per group). For Turkey's post hoc analyses—$^{\#}$Sco vs. Sco + PIM1% $p < 0.0001$ and $^{\$}$Sco vs. Sco + PIM3% $p < 0.001$ (**a**) and $^{\#}$Sco vs. Sco + PIM1% $p < 0.0001$ and $^{\$}$Sco vs. Sco + PIM3% $p < 0.001$ (**b**)

$r = 0.473$, $p < 0.01$) (Fig. 6b), the percentage of the spontaneous alternation vs. immobility time ($n = 24$, $r = -0.466$, $p < 0.01$) (Fig. 6c), reference memory errors vs. swimming time ($n = 24$, $r = -0.512$, $p < 0.01$) (Fig. 6d) and reference memory errors vs. immobility time ($n = 24$, $r = 0.476$, $p < 0.01$) (Fig. 6e) were evidenced.

Discussion

In the present study, we investigated whether inhalation of the *P. peregrina* essential oil (PIM1% and PIM3% for 21 continuous days) causes behavioral effects as memory-enhancing, anxiolytic, and antidepressant-like effects in scopolamine-treated rats based on specific behavioral tests (Y-maze, radial arm-maze, EPM, and FST).

Scopolamine is thought to exert various toxic properties on the nervous system. It exhibited toxicity on the population and dendritic development of the newborn neurons and immature granular cells in dentate gyrus, which directly results in injury of the hippocampal circuits that may predominantly be responsible for cognitive and memory deficits [22]. Inhibition of the muscarinic acetylcholine receptor by scopolamine also contributes to characteristic cognitive and memory deficits of AD [23], as well as the cholinergic receptor antagonists [24].

In the present study, we used two well-characterized hippocampus-dependent spatial memory tasks: Y-maze and radial arm-maze. These behavioral tasks can test hippocampus-dependent short-term, long-term, and spatial memory processing, which are particularly affected by AD [25].

The GC-MS/GC-FID analyses indicated *trans*-pinocarveol (35.1 %), followed by pregeijerene (15.1 %), α-cubebene (12.4 %), (+)-epi-bicyclosesquiphellandrene (7.5 %), α-terpineol (6.7 %), allocymene (4.0 %), iso-spathulenol (3.1 %), and bicyclogermacrene (1.8 %), as the main components of our *P. peregrina* essential oil suggesting that these constituents could be responsible for the observed behavioral effects in scopolamine-treated rats. Previous reports indicated that monoterpens hydrocarbons have marked effects on the memory formation by modulating the glutamate activation in vitro and in vivo by competitive antagonism of ionotropic receptors of the type N-methyl-D-aspartate (NMDA) [26]. Glutamate is an excitatory neurotransmitter that drives changes in synaptic activity associated with mechanisms responsible for memory formation [27]. It is known that NMDA receptors are crucial for the induction of activity-dependent synaptic plasticity and memory formation. Extensive evidence shows that NMDA receptors play an important role in memory formation to aversive conditioning, spatial and nonspatial memory training, and nonaversive tasks [28]. In the light of these studies, we presume that our high-*trans*-pinocarveol (35.1 %) containing essential oil sustains spatial memory formation in scopolamine-induced amnesia in the specific behavioral tests (Y-maze, radial arm-maze).

The Y-maze task is a specific and sensitive test of spatial recognition memory in rodents. The test relies on an innate tendency of rats to explore a novel environment. The Y-maze used in this study involves no aversive stimuli and was considered suitable for evaluating memory. The specific part of the brain involved in the performance of this task includes the hippocampus [17].

The high dose (PIM3%) of *P. peregrina* essential oil in scopolamine-treated rats significantly improved spatial working memory, as evidenced by the increase of spontaneous alternation percentage as compared to scopolamine alone-treated rats. This result suggests that the high dose of *P. peregrina* essential oil (PIM3%) used in this study displays an improved effect on acquisition of the short-term memory of scopolamine-treated rats within the Y-maze task. However, no differences were observed between both doses of *P. peregrina* essential oil on spatial working memory within the Y-maze task. The

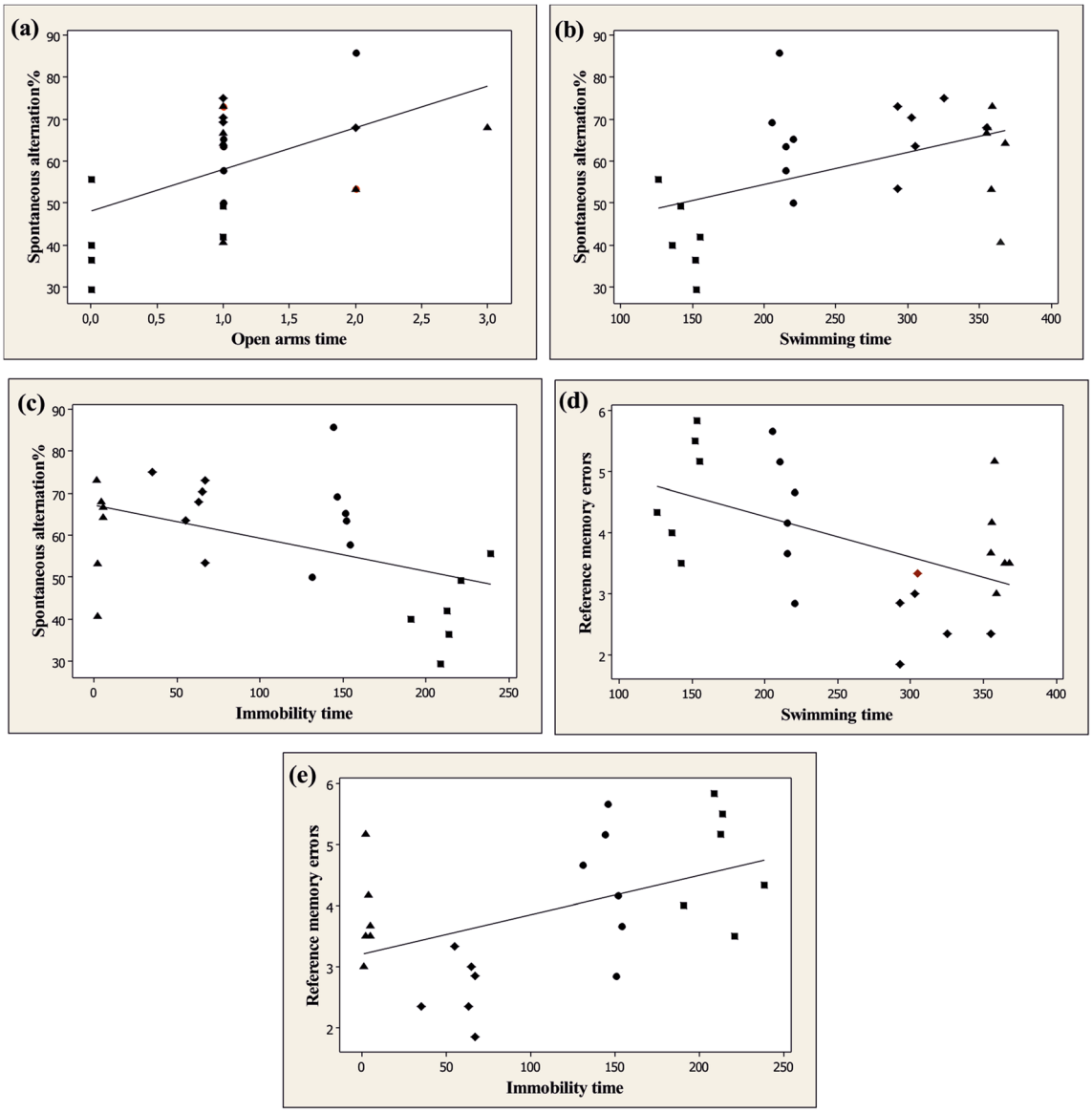

Fig. 6 Pearson's correlation between spontaneous alternation % vs. open arms time (**a**), spontaneous alternation % vs. swimming time (**b**), spontaneous alternation % vs. immobility time (**c**), reference memory errors vs. swimming time (**d**), and reference memory errors vs. immobility time (**e**) in control group (*closed circle*), scopolamine (Sco) alone-treated group (*closed square*), Sco + PIM1% group (*closed triangle*) and Sco + PIM3% group (*closed diamond*)

improvement of spatial working memory within Y-maze task cannot be attributed to locomotor activity, because significant changes in the number of entries of the groups treated with the *P. peregrina* essential oil as compared with scopolamine alone-treated rats were observed.

In the behavioral neuroscience trial, radial arm-maze task is widely used [29]. The radial arm-maze is one of the standard apparatuses used in behavioral-based research, commonly using rats as experimental animals to assess spatial memory. Spatial memory, either its working memory and/or reference memory components, refers to memory for spatial information by which the brain works in recognizing, codifying, storing, and recovering information about objects or routes. The spatial memory recognitions have always been related to exploratory behavior and curiosity. This kind of behavior may represent the need to acquire information when subjects face new environments [30].

Thus, scopolamine-treated rats exposed to both doses of *P. peregrina* essential oil (PIM1% and PIM3%) exhibited an improvement of working memory along with an improvement of long-term memory, explored by reference memory as compared with scopolamine alone-treated rats within the radial arm-maze task. These findings suggest that inhalation of the *P. peregrina* essential oil plays an important role in spatial memory formation, especially on working memory and reference memory. However, non-significant differences were

observed between both doses of the *P. peregrina* essential oil on working memory and reference memory in the radial arm-maze task.

The present study also evaluated whether memory impairment induced by scopolamine is related with anxiety and depressive behaviors as assessed in specific behavioral tests (elevated plus-maze and forced swimming test).

The elevated plus-maze is recognized as a valuable model able to predict anxiolytic- or anxiogenic-like effects of drugs in rodents [31]. Scopolamine alone-treated rats have decreased the percentage of the time spent in the open arms, the number of open-arm entries and the number of crossing (exploratory activity) in the elevated plus-maze test. This indicates that the scopolamine-alone treated rats experienced high levels of anxiety and were suitable for evaluating the presumed anxiolytic substances as our essential oil [18].

In the present study, we demonstrated that inhalation of *P. peregrina* essential oil in scopolamine-treated rats produces anxiolytic-like activity. Furthermore, after the scopolamine-treated rats being exposed to *P. peregrina* essential oil, the percentage of time spent in the open arms significantly increased, especially in the group exposed to 1 % essential oil as compared to scopolamine-alone treated rats. Additionally, the number of open arms entries increased, especially in the group of scopolamine-treated rats exposed to *P. peregrina* essential oil 1 % as compared to scopolamine-alone treated rats.

As expected, diazepam (DZP) as a benzodiazepine drug used as positive control produced significant increases in the percentage of time spent in the open arms, the number of open-arm entries, and the number of crossing (exploratory activity) as compared to scopolamine-alone treated rats. These data are consistent with the results of numerous previous studies, which have shown that DZP and other benzodiazepines produce significant anxiolytic effects in a variety of anxiolytic screening procedures, including elevated plus-maze test procedures [32]. The pharmacological action of diazepam enhances the effect of the neurotransmitter GABA by binding to the benzodiazepine site on the $GABA_A$ receptor (via the constituent chlorine atom) leading to central nervous system (CNS) depression [33]. The anxiety indicators in the elevated plus-maze (the percentage of the time spent in the open arms and the number of open-arm entries) showed up being sensitive to the agents which were thought to act via the $GABA_A$ receptor complex [34]. It has been reported that various terpenes, including *trans*-pinocarveol, carvacrol, pinene, and β-caryophyllene from *Sideritis* extracts, could modulate the $GABA_A$ receptors activity [35]. Thus, terpenes with distinct structural properties may mediate sedative or anxiolytic mechanisms involving $GABA_A$ receptors.

In light with these reports, our high-*trans*-pinocarveol (35.1 %), pregeijerene (15.1 %), and α-cubebene (12.4 %) containing *P. peregrina* essential oil have increased anxiolytic-like behavior and anti-depressive-like response in scopolamine-treated rats.

The forced swimming test has been validated as a suitable tool for predicting the antidepressant properties of drugs [29]. When rodents are forced to swim in a confined space, after an initial period of struggling, they would become immobile, resembling a state of despair and mental depression. This inescapable stressful situation can be evaluated by assessing different behavioral strategies [36].

The swimming time decreased and the immobility time increased in scopolamine alone-treated rats as compared to control rats. This indicates that the scopolamine alone-treated rats exhibited depression. After being exposed to both doses of *P. peregrina* essential oil (PIM1% and PIM3%), the swimming time significantly increased, especially in group exposed to PIM1% essential oil as compared to scopolamine alone-treated rats. Moreover, the decrease of the immobility time, especially in group exposed to PIM1% essential oil as compared to scopolamine alone-treated rats, was also observed.

These results suggested that *P. peregrina* essential oil possesses a strong antidepressant-like response to an inescapable stress. In our study, tramadol (TRM), as positive control, produced significant increases in the swimming time and decreases the immobility time as compared to scopolamine-alone treated rats. Tramadol is a unique drug with multiple modes of action. It is a weak agonist of the μ-opioid receptor but it also inhibits the reuptake of serotonin as well as norepinephrine. It is an analgesic and it is also considered as an antidepressant [37].

Moreover, we found a significant correlation between the percentage of the spontaneous alternation vs. the number of the open arm entries, the percentage of the spontaneous alternation vs. swimming time, the percentage of the spontaneous alternation vs. immobility time, reference memory errors vs. swimming time and reference memory errors vs. immobility time when linear regression was determined.

These data suggest that memory-enhancement scores within Y-maze and radial arm-maze tests along with decrease of anxiety and depressive-like behaviors within elevated plus-maze and forced swimming tests could be related with the involvement of the inhaled *P. peregrina* essential oil against scopolamine-induced memory impairment, anxiety and depression in laboratory rats.

Conclusion

Taken together, our findings suggest that *P. peregrina* essential oil exhibited memory-improving, anxiolytic, and antidepressant effects in scopolamine-treated rats. In conclusion, inhalation of the *P. peregrina* essential oil might offer a useful alternative or complementary choice in either the prevention

or the treatment of psychiatric condition close related to AD conditions.

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Compliance with Ethical Standards Rats were treated in accordance with the guidelines of the animal bioethics of the Act on Animal Experimentation and Animal Health and Welfare from Romania and all procedures were in compliance with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. This study was approved by the Committee on the Ethics of Animal Experiments of the Alexandru Ioan Cuza University of Iasi (Permit Number: 2192) and also, efforts were made to minimize animal suffering and to reduce the number of animals used.

Conflict of Interest The authors declare that they have no competing interests.

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