Anxiolytic and antidepressant-like effects of *Ferulago angulata* essential oil in the scopolamine rat model of Alzheimer’s disease

Eyup Bagci, Emel Aydin, Marius Mihasan, Calin Maniu and Lucian Hritcu

Abstract: *Ferulago angulata* subsp. *carduchorum* (Apiaceae) is a shrub indigenous to western Iran, Turkey and Iraq. In traditional medicine, *F. angulata* is recommended for treating digestive pains, haemorrhoids, snake bites, ulcers and as a sedative. The present study analysed the possible anxiolytic, antidepressant and antioxidant properties of *F. angulata* essential oil in a scopolamine-induced rat model of Alzheimer’s disease. The anxiolytic and antidepressant-like effects of *F. angulata* essential oil were studied using *in vivo* (elevated plus-maze and forced swimming tests) approaches. Also, the antioxidant activity in the amygdala was assessed using superoxide dismutase, glutathione peroxidase and catalase specific activities, the total content of the reduced glutathione, protein carbonyl and malondialdehyde levels. The scopolamine-treated rats exhibited the following: a decrease in the percentage of the time spent and the number of entries in the open arm within the elevated plus-maze test and a decrease of swimming time and an increase of immobility time in the forced swimming test. Inhalation of *F. angulata* essential oil significantly exhibited anxiolytic and antidepressant-like effects and also antioxidant potential. Furthermore, *in silico* studies carried out by employing molecular docking experiments pointed to the existence of strong interactions of monoterpenes from *F. angulata* essential oil with anxiolytic and antidepressant effects with GABA<sub>A</sub> receptor. Our results suggest that the *F. angulata* essential oil inhalation ameliorates scopolamine-induced anxiety and depression by attenuation of the oxidative stress in the rat amygdala. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: *Ferulago angulata* essential oil; anxiety; depression; oxidative stress; Alzheimer’s disease

Introduction

Alzheimer’s disease (AD) is the most prevalent form of a neurodegenerative disorder and constitutes approximately two-thirds of all cases of dementia. The hallmark pathology of AD comprised of widespread amyloid-beta (Aβ) deposition, neurofibrillary tangle formation and extensive neurodegeneration in the brain. AD pathology is formed by the complex interaction between multiple genetic and environmental factors. Currently, no effective treatments for AD are available. Progressive cognitive and behavioural impairment is characteristic in AD. There is evidence indicating that the pathological process begins years if not decades before clinical symptoms occur.

Although cognitive symptoms are characteristic of AD, non-cognitive symptoms are becoming increasingly important because of the prevalence and dysfunctions they generate. Non-cognitive symptoms, such as agitation, aggression, depression, anxiety and psychosis are often observed in AD patients. These symptoms known as ‘behavioural and psychological symptoms of dementia’ (BPSD) have been reported to occur in about 20% of AD patients. These symptoms increase the caregiver stress and impairment in daily living activities, worsen the patient’s quality of life and also accelerate cognitive decline.

The amygdala is one of the early structures to undergo neurodegeneration in AD. It is one of the structures where tau deposition occurs in the earliest stages of AD pathology. The amygdala also has abundant neuronal connections with the hippocampus. It has been shown that substantial atrophy within the amygdala in AD was evidenced. It is associated with a role in emotional processing and the storage of emotional memories. The amygdala is the key structure in the acquisition and expression of fear, influencing affective states such as depression and anxiety. It is known that amygdala lesions have deleterious consequences on primate social behaviour.

The involvement of GABA neurotransmission in the mediation of fear and anxiety has been well-documented. Benzodiazepines may interfere with both the acquisition and expression of learned fear by potentiating the inhibitory effects of GABA in the basolateral amygdala (BLA). A decline in GABA<sub>B</sub> receptor signaling triggers hyperactive neurological disorders such as insomnia, anxiety and epilepsy. In response to binding the neurotransmitter GABA, released at inhibitory synapses, GABA<sub>B</sub> receptor chloride channels open and depress neuronal excitability in the adult central nervous system. Also, the identification of the crystal structure of this type of receptor is a potential opportunity in *in silico* studies regarding molecular docking.

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Although the causative factors of AD remain unclear, there is growing evidence suggesting that oxidative stress plays an important role in the pathogenesis of the disease. Several studies reported that DNA, RNA, lipid and protein oxidation are present in the brains of AD patients, suggesting that oxidative stress is an early event in AD pathogenesis.

Scopolamine is a blocker of the muscarinic acetylcholine receptor that serves as a beneficial pharmacological tool in producing a model of amnesia. Scopolamine is used as a standard/reference drug for inducing cognitive deficits in healthy humans and animals, it is thought to exert various toxic properties on the nervous system and also, exhibited toxicity in 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. Inhibition of the muscarinic acetylcholine receptor by scopolamine also contributes to characteristic cognitive and memory deficits of AD as well as the cholinergic receptor antagonists. There is notable evidence that scopolamine causes oxidative stress through the interference with acetylcholine in the brain leading to cognitive impairment.

_Ferula angulata_ L. is widespread in the high altitudes of several Asian countries such as Iraq and Iran as well as Turkey. The genus _Ferula_ is represented by 50 species worldwide and 31 of which are found in Turkey. _Ferula angulata_ was divided into two subspecies by Chamberlain in 1987. The known subspecies distributed in Turkey is _F. angulata_ subsp. _carduchorum_ differs from subsp. _angulata_ by having scabrid inflorescence, ovrium and leaves (not glabrous or subglabrous).

In folk medicine, different species of _Ferula_ has been used in Turkey and Iran as sedative, tonic and remedy of digestive panics, aphrodisic properties and haemorrhoids. Moreover, different parts of _Ferula_ species have been traditionally used against ulcers, snake bite and for the treatment of a headache and disease of rescence, ovarium and leaves (not glabrous or subglabrous).

The essential oils of _Ferula assafoetida_ and _Ferula vulgaris_ species distributed in Turkey is _F. angulata_ subsp. _carduchorum_. The essential oil of _F. angulata_ subsp. _carduchorum_ was evaluated for its potential as an alternative to the standard/reference drug for inducing cognitive deficits in animals. The essential oil was diluted with 1% Tween 80 (v/v). DZP-, TRM- and scopolamine alone-treated animals, which were individually exposed to 0.9% saline with 1% Tween 80 treatment, as a negative control; (2) the DZP-treated group received 0.9% saline with 1% Tween 80 treatment; (3) Diazepam alone-treated group (DZP, 1.5 mg/kg) received 0.9% saline with 1% Tween 80 treatment, as a positive control; (4) Tramadol alone-treated group (TRM, 10 mg/kg) received 0.9% saline with 1% Tween 80 treatment, as a positive control; (5) the Scopolamine-treated group received _F. angulata_ essential oil 1% (Sco+FRG1%) and (6) the Scopolamine-treated group received _F. angulata_ essential oil 3% (Sco+FRG3%). The Control, DZP, TRM- and Sco 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. All experimental procedures were performed in compliance with the animal use regulations of Firat University, Elazig, Turkey. This study was approved by the Committee on the Ethics of Laboratory Animal Experiments of the Firat University, Elazig, Turkey (permit number: 147/02.07.2014) and also, efforts were made to minimize animal suffering and to reduce the number of animal used. No animals died during the behavioural tests.

### Inhalation apparatus and drug administration

The inhalation apparatus consisted of a Plexiglass chamber (50 × 40 × 28 cm). Two chambers were used, one for the control, DZP-, TRM- and scopolamine alone-treated animals, which were individually exposed to 0.9% saline with 1% Tween 80 solution and the other one for the experimental animals, which were individually exposed to _F. angulata_ essential oil. _Ferula angulata_ essential oil was diluted with 1% Tween 80 (v/v). _Ferula angulata_ essential oil exposure (200 µl, either 1% or 3%) was via an electronic vaporizer placed at the bottom of the chamber, but out of reach of the animals. Rats in the _F. angulata_ essential

### Material and methods

#### Plant materials and _F. angulata_ essential oil preparation

Aerial parts of _F. angulata_ subsp. _carduchorum_ were collected in the flowering stage in Bingol, Eastern Anatolia, Turkey, in June 2013 and identified by Dr Eyup Bagci at the Herbarium of Department of Biology, Firat University where a voucher specimen was registered and deposited for ready reference. Air-dried aerial parts of the plant samples were subjected to hydro-distillation for 3 h using a Clevenger-type apparatus to obtain the essential oil. The total essential oil yield was 0.7 % (v/w).

#### Gas chromatography (GC-MC/GC-FID) analysis

GC-MS analysis of the _F. angulata_ subsp. _carduchorum_ essential oil was performed in Plant Products and Biotechnology Research Laboratory (BUBAL), Firat University, using Hewlett-Packard- Agilent 5973 N GC-MS system (Agilent Technologies, USA) with 6890 GC equipped with a flame ionization detector (FID). HP-5 MS column [30 m × 0.25 mm i.d., the film thickness (0.25 µm)] was used with helium as the carrier gas. The injector temperature was 250 °C; the 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 for 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 a comparison of their RI, their retention times (RT) and mass spectra with those obtained from authentic Wiley libraries (available from Hewlett-Packard) and the literature.

#### 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) the Control group received 0.9% saline with 1% Tween 80 treatment; (2) the Scopolamine (Sco) alone-treated group received 0.9% saline with 1% Tween 80 treatment, as a negative control; (3) Diazepam alone-treated group (DZP, 1.5 mg/kg) received 0.9% saline with 1% Tween 80 treatment, as a positive control; (4) Tramadol alone-treated group (TRM, 10 mg/kg) received 0.9% saline with 1% Tween 80 treatment, as a positive control; (5) the Scopolamine-treated group received _F. angulata_ essential oil 1% (Sco+FRG1%) and (6) the Scopolamine-treated group received _F. angulata_ essential oil 3% (Sco+FRG3%). The Control, DZP, TRM- and Sco 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. All experimental procedures were performed in compliance with the animal use regulations of Firat University, Elazig, Turkey. This study was approved by the Committee on the Ethics of Laboratory Animal Experiments of the Firat University, Elazig, Turkey (permit number: 147/02.07.2014) and also, efforts were made to minimize animal suffering and to reduce the number of animal used. No animals died during the behavioural tests.

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oil groups were exposed to oil vapours for a 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 a 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 behavioural testing. Diazepam (Sigma-Aldrich) and tramadol hydrochloride (Sigma-Aldrich) were used as positive controls and were injected i.p. in a volume of 1 ml/kg in laboratory rats, 1 h before behaviourally tested.

**In silico docking experiments**

For docking studies, the file PDB ID: 4COF from RCSB PDB was used. The molecular docking procedure was carried out using VEGA ZZ, a complete molecular modelling suite in conjunction with NAMD and AutoDock4.

To conduct simulations of macromolecular structures such as Energy Minimization and Ligand-Receptor Docking, explicit hydrogen atoms are required for all-atom molecular mechanics, docking and electrostatic calculations. The protonation state problem exists not only with low-resolution X-ray structures but also with high-resolution structures. This implies a careful refinement of the PDB structure beginning with normalizing the coordinates to translate the protein at the origin of the Cartesian axis followed by the addition of the hydrogens. Subsequently, the few remaining protonation problems have been identified and corrected by manual means. To optimize the crystal structure of the complex, an energy minimization was performed with NAMD.

The GABA receptor has five neurotransmitter-binding sites. To generate complexes in which the ligand is placed in the same pocket of the co-crystallized one, the atoms selected included a 10Å sphere around the first binding site. The co-crystallized ligand was removed to create enough space to dock the new molecules. The compounds of the F. angulata essential oil selected for docking were as follow: α-pinene (CID 6654), β-pinene (CID 14896), β-phellandrene (CID 11142), α-phellandrene (CID 7460), p-cymene (CID 7463), β-myrcene (CID 31253), 2,5-diethylthiophene (CID 521294) and sabinene (CID 18818). To assess the results, the agonist molecules with a known effect were also tested: GABA (Sigma-Aldrich) and sabinene (CID 18818). To assess the results, the protonation state problem exists not only with low-resolution X-ray structures but also with high-resolution structures. This implies a careful refinement of the PDB structure beginning with normalizing the coordinates to translate the protein at the origin of the Cartesian axis followed by the addition of the hydrogens. Subsequently, the few remaining protonation problems have been identified and corrected by manual means. To optimize the crystal structure of the complex, an energy minimization was performed with NAMD.

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**Behavioural tests**

*Elevated plus-maze test*

Behaviour in the elevated plus-maze (EPM) is also utilized to assess exploration, anxiety and motor behaviour. 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. Fifteen minutes after the inhalation of F. angulata essential oil (FRG1% and FRG3%), each rat was placed in the centre of the maze facing one closed arm. Behaviour was observed for 5 min, and the time spent and a number of entries into the open and enclosed arms were counted. The percentages of time spent in the open arms (time spent in the open arms/time spent in all arms × 100) were calculated. An entry was defined as an animal placing all four paws on 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 (FST)**

The FST is the most widely used model for assessing a depressive-like response. The depressive-like response was assessed, basically using the same method described by Campos et al., 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 F. angulata essential oil (FRG1% and FRG3%). 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).

**Biochemical parameter assay**

After the behavioural tests, all rats were deeply anaesthetized (using sodium pentobarbital, 100 mg/kg b.w., i.p.; Sigma-Aldrich) and decapitated and whole brains were removed. The bilateral amygdala was carefully excised. Each of the amygdala samples was weighted and homogenized (1:10) with Potter Homogenizer coupled with Cole-Parmer Servodyne Mixer in ice-cold 0.1 M potassium phosphate buffer (pH 7.4), 1.15% KCl. The homogenate was centrifuged (15 min at 960 g) and the supernatant was used for assays of SOD, CAT, GPX-specific activities, the total content of reduced GSH, protein carbonyl and MDA levels.

**Determination of amygdala SOD activity**

The activity of superoxide dismutase (SOD, EC 1.15.1.1) was assayed by monitoring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). Each 1.5 ml reaction mixture contained 100 mM TRIS/HCl (pH 7.8), 75 mM NBT, 2 μM riboflavin, 6 mM EDTA and 200 μl of the supernatant. Monitoring the increase in absorbance at 560 nm followed the production of blue formazan. One unit of SOD is defined as the quantity required to inhibit the rate of NBT reduction by 50% as previously described by Winterbourn et al. The enzyme activity is expressed as units/mg protein.

**Determination of amygdala CAT activity**

Catalase (CAT, EC 1.11.1.6) activity was assayed according to the method of Sinha. The reaction mixture consisted of 150 μl phosphate buffer (0.01 M, pH 7.8), 100 μl supernatants. The reaction was started by adding 250 μl H2O2 0.16 M, incubated at 37 °C for 1 min, and the reaction was stopped by addition of 1 ml of dichromate: acetic acid reagent. The tubes were immediately kept in a boiling water bath for 15 min and the green colour developed while the reaction was read at 570 nm on a spectrophotometer. Control tubes, devoid of the enzyme, were also processed in parallel. The enzyme activity is expressed as μmol of H2O2 consumed/min/mg protein.
Glutathione peroxidase activity

The extent of protein oxidation in the amygdala was assessed by a spectrophotometric assay. A reaction mixture consisting of 1 ml of 0.4 M phosphate buffer (pH 7.0) containing 0.4 mM EDTA, 1 ml of 5 mM NaN₃, 1 ml of 4 mM glutathione (GSH) and 200 μl of supernatant was pre-incubated at 37 °C for 5 min. Then 1 ml of 4 mM H₂O₂ was added and incubated at 37 °C for a further 5 min. The excess amount of GSH was quantified by the 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) method as previously described by Sharma and Gupta.⁴⁶ One unit of GPX is defined as the amount of enzyme required to oxidize 1 nmol GSH/min. The enzyme activity is expressed as units/mg protein.

Total amygdala content of reduced GSH

Glutathione (GSH) was measured according to the method of Fukuzawa and Tokumura.⁴⁷ Next, 200 μl of supernatant was added to 1.1 ml of 0.25 M sodium phosphate buffer (pH 7.4) followed by the addition of 130 μl DTNB 0.04%. Finally, the mixture was brought to a final volume of 1.5 ml with distilled water, and the absorbance was read in a spectrophotometer at 412 nm and the results were expressed as μg GSH/μg protein.

Determination of amygdala protein carbonyl level

The extent of protein oxidation in the amygdala was assessed by measuring the content of the protein carbonyl groups, using 2,4-dinitrophenylhydrazine (DNPH) derivatization as described by Fukuzawa and Tokumura.⁴⁷ The supernatant fraction was divided into two equal aliquots containing approximately 2 mg of protein each. Both aliquots were precipitated with 10% trichloroacetic acid (TCA, w/v, final concentration). One sample was treated with 2 N HCl, and the other sample was treated with an equal volume of 0.2% (w/v) DNPH in 2 N HCl. Both samples were incubated at 25 °C and stirred at 5-min intervals. The samples were then reprecipitated with 10% TCA (final concentration) and subsequently extracted with ethanol-ethyl acetate (1:1, v/v) and then reprecipitated with 10% TCA (final concentration) and subsequently extracted with ethanol-ethyl acetate (1:1, v/v) and then reprecipitated with 10% TCA. The pellets were carefully drained and dissolved in 6 M guanidine hydrochloride with 20 mM sodium phosphate buffer, pH 6.5. The insoluble debris was removed by centrifugation at 13,000 g for 40 min. Afterwards, samples were centrifuged at 960 g for 10 min and the supernatants were read at 532 nm. A calibration curve was constructed using MDA as a standard, and the results were expressed as nmol/mg protein.

Malondialdehyde (MDA), which is an indicator of lipid peroxidation, was spectrophotometrically measured using the thiobarbituric acid assay as previously described by Ohkawa et al.⁵⁰ In total, 200 μl of supernatant was added and briefly mixed with 1 ml of 50% trichloroacetic acid in 0.1 M HCl and 1 ml of 26 mM thiobarbituric acid. After vortex mixing, samples were maintained at 95 °C for 10 min. Afterwards, samples were centrifuged at 960 g for 10 min and the supernatants were read at 532 nm. A calibration curve was constructed using MDA as a standard, and the results were expressed as nmol/mg protein.

Statistical analysis

Behavioural scores within elevated plus-maze and forced swimming tests and biochemical data were analysed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test using GraphPad Prism 6 software for Windows, La Jolla, CA, USA. All results are expressed as the mean ± standard error of mean (SEM). F-values for which P < 0.05 were regarded as statistically significant.

Results

Chemical composition of the F. angulata essential oil

The chemical composition of the F. angulata subsp. carduchorum essential oil obtained from a single plant species may exhibit seasonal and geographical variability and was analysed by GC-MS/GC-FID. The chemical composition (%) of identified compounds in the essential oil of F. angulata subsp. carduchorum aerial parts is listed in Table 1. A total of 48 different compounds were isolated which constituted 96.5 % (w/w) of the total essential oil. The principal components of the essential oil were monoterpene hydrocarbons (C₁₀H₁₆), including α-pinene (24.10%), β-pinene (22.70%), α-phellandrene (12.10%) and β-phellandrene (20.50%), which accounted for 79.40% of the total essential oil.
Docking studies of receptor-ligand interactions

The GABA<sub>A</sub> receptor-β3<sub>1</sub>cryst is a homopentamer and has five similar conformation neurotransmitter binding sites located between the extracellular domains. In the 4COF model, the benzamidine agonist occupies all these pockets. The benzamidine benzyl ring is stacked between the side chains of Phe 200 and Tyr 62. For this reason, the molecular docking studies have been made on the first binding pocket, which was prepared properly. To predict the appropriate interaction of diazepam, benzamidine, GABA and F. angulata essential oil compounds with the GABA<sub>A</sub> receptor, the ligand molecules were docked with the target protein using VEGA ZZ in conjunction with GridDock/AutoDock4. Every docking contains some useful knowledge that includes the inhibition constant (ki), the free energy of binding, total intermolecular energy, final total internal energy, torsional free energy and unbound system's energy. All docking results indicate that benzamidine, diazepam, α-pinene, β-pinene, α-phellandrene and β-phellandrene ligands with the receptor protein, GABA<sub>A</sub>, gave the best docking results. According to Table 2, these compounds present the smaller value of ki and the lowest amount of free energy of binding as compared with diazepam. In the Figure 1, the best docking position of diazepam (Figure 1a), GABA (Figure 1b), α-pinene (Figure 1c), β-pinene (Figure 1d), α-phellandrene (Figure 1e) and β-phellandrene (Figure 1f) on the GABA<sub>A</sub> receptor are shown.

Effect of the F. angulata essential oil on elevated plus maze behavior

As can be seen in Figure 2a, in the elevated plus-maze task ANOVA, revealed a significant overall effect [F(4,25) = 6.50, P < 0.001] on the percentage of the time spent in the open arms. Additionally, Tukey's post hoc analysis revealed a significant difference between the control vs. the Sco groups (P < 0.01), the DIAZ vs. the Sco groups (P < 0.01), the Sco vs. the Sco+FRG1% groups (P < 0.01), the Sco vs. the Sco+FRG3% groups (P < 0.0001) and the Sco + FRG1% vs. the Sco + FRG3% groups (P < 0.01) for the percentage of the time spent in the open arms (Figure 2a).

As can be seen in Figure 2b, in the elevated plus-maze task ANOVA revealed a significant overall effect [F(4,25) = 5.61, P < 0.001] on the number of open-arm entries. Additionally, Tukey's post hoc analysis revealed a significant difference between the control vs. the Sco groups (P < 0.01), the DIAZ vs. the Sco + FRG1% groups (P < 0.01), the Sco vs. the Sco + FRG1% groups (P < 0.01), the Sco vs. the Sco + FRG3% groups (P < 0.0001) and the Sco + FRG1% and the Sco + FRG3% groups (P < 0.01) for the number of open-arm entries (Figure 2b).

Effect of the F. angulata essential oil in the rat forced swimming test

In the forced swimming test, ANOVA revealed a significant overall effect on the swimming time [F(4,25) = 55.28, P < 0.0001] (Figure 2c) and on the immobility time [F(4,25) = 65.85, P < 0.0001] (Figure 2d). Additionally, Tukey's post hoc analysis revealed a significant difference between the control vs. the Sco groups (P < 0.0001), the control vs. the Sco + FRG3% groups (P < 0.0001), the TRM vs. the Sco groups (P < 0.0001), the TRM vs. the Sco + FRG1% groups (P < 0.01), the TRM vs. the Sco + FRG3% groups (P < 0.01), the Sco vs. the Sco + FRG1% groups (P < 0.0001), the Sco vs. the Sco + FRG3% groups (P < 0.0001) and the Sco + FRG1% and the Sco + FRG3% groups (P < 0.01) for the swimming time.

Table 1. Chemical composition (%) of identified compounds in the essential oil of Ferulago angulata subsp. carduchorum aerial parts

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<th>No.</th>
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RI<sup>a</sup>, literature retention indices.
RI<sup>b</sup>, experimental retention indices relative to n-alkanes on the HP-5 MS column.
### Table 2. Ligand–protein interaction parameters

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<tr>
<th>Molecule</th>
<th>Inhibition constant, $K_i$ (μM)</th>
<th>Free energy of binding (kcal/mol)</th>
<th>Total intermolecular energy (kcal/mol)</th>
<th>Final total internal energy (kcal/mol)</th>
<th>Torsional free energy (kcal/mol)</th>
<th>Unbound system’s energy (kcal/mol)</th>
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**Figure 1.** The binding surface and the docking position for molecule (a) diazepam (CID 3016), (b) GABA (CID 119), (c) α-pinene (CID 6654), (d) β-pinene (CID 14896), (e) α-phellandrene (CID 7460) and β-phellandrene (CID 11142) as depicted in magenta.
Moreover, Tukey’s post hoc analysis revealed a significant difference between the control vs. the Sco groups ($P < 0.0001$), the control vs. the Sco+FRG1% groups ($P < 0.0001$), the control vs. the Sco+FRG3% groups ($P < 0.0001$), the TRM vs. the Sco groups ($P < 0.0001$), the TRM vs. the Sco+FRG3% groups ($P < 0.0001$), the Sco vs. the Sco+FRG1% groups ($P < 0.0001$), the Sco vs. the Sco+FRG3% groups ($P < 0.0001$) and the Sco+FRG1% and the Sco+FRG3% groups ($P < 0.001$) for the immobility time (Figure 2d).

For SOD-specific activity estimated in the rat amygdala homogenates, ANOVA revealed a significant overall effect $[F(3,36) = 17.37, P < 0.0001]$ (Figure 3a). Additionally, Tukey’s post hoc analysis revealed significant differences between the control vs. the Sco groups ($P < 0.001$), the Sco vs. the Sco+FRG1% groups ($P < 0.001$) and the Sco vs. the Sco+FRG3% groups ($P < 0.001$) for SOD specific activity (Figure 3a).

For GPX-specific activity estimated in the rat amygdala homogenates, ANOVA revealed a significant overall effect $[F(3,36) = 6.44, P < 0.01$ (Figure 3b). Additionally, Tukey’s post hoc analysis revealed significant differences between the control vs. the Sco groups ($P < 0.01$), the Sco vs. the Sco+FRG1% groups ($P < 0.01$) and the Sco vs. the Sco+FRG3% groups ($P < 0.01$) for GPX-specific activity (Figure 3b).

For CAT-specific activity estimated in the rat amygdala homogenates, ANOVA revealed a significant overall effect $[F(3,36) = 13.61, P < 0.001$ (Figure 3c). Additionally, Tukey’s post hoc analysis revealed significant differences between the control vs. the Sco groups ($P < 0.001$), the control vs. the Sco+FRG1% groups ($P < 0.01$), the control vs. the Sco+FRG3% groups ($P < 0.01$), the Sco vs. the Sco+FRG1% groups ($P < 0.01$) and the Sco vs. the Sco+FRG3% groups ($P < 0.01$) for CAT-specific activity (Figure 3c).

For the total content of reduced GSH estimated in the rat amygdala homogenates, ANOVA revealed a significant overall effect $[F(3,36) = 44.45, P < 0.0001]$ (Figure 3d). Additionally, Tukey’s post hoc analysis revealed significant differences between the control vs. the Sco groups ($P < 0.0001$), the Sco vs. the Sco+FRG1% groups ($P < 0.0001$) and the Sco vs. the Sco+FRG3% groups ($P < 0.0001$) for the total content of reduced GSH (Figure 3d).

For the protein carbonyl levels estimated in the rat amygdala homogenates, ANOVA revealed a significant overall effect $[F(3,36) = 51.62, P < 0.0001$ (Figure 3e). Additionally, Tukey’s post hoc analysis revealed significant differences between the control vs. the Sco groups ($P < 0.0001$), the Sco vs. the Sco+FRG1% groups ($P < 0.0001$) and the Sco vs. the Sco+FRG3% groups ($P < 0.0001$) for the protein carbonyl level (Figure 3e).
For the MDA levels estimated in the rat amygdala homogenates, ANOVA revealed a significant overall effect \( F(3, 36) = 40.63, P < 0.0001 \) (Figure 3f). Additionally, Tukey's post hoc analysis revealed significant differences between the control vs. the Sco groups \( (P < 0.0001) \), the Sco vs. the Sco+FRG1% groups \( (P < 0.0001) \) and the Sco vs. the Sco+FRG3% groups \( (P < 0.0001) \) for the MDA level (Figure 3f). These findings support the hypothesis that the *F. angulata* volatile oil may have induced a decrease in neuronal oxidative stress.

**Effect of the *F. angulata* essential oil on DNA fragmentation**

In our study, DNA cleavage patterns were absent in the *F. angulata* essential oil groups (Figure 4), implying that *F. angulata* essential oil do not induced apoptotic events.

**Discussion**

The use of aromatic plants to relief different illness is not a new therapy. Actually aromatic plants have been used for many centuries by different cultures around the world, including Turkey. Pharmacological studies provide scientific support to the traditional use of aromatic medicinal plants and aromatherapy; nevertheless, more clinical trials are required regarding to their effectiveness in order to establish a guidance for their use in routine healthcare.\(^{[53]}\)

The present study was aimed to examine the anxiety and depressive-like response after inhalation of the *F. angulata* essential oil (200 μl, 1% and 3%) in rats subjected to injection of scopolamine. Consequently, injection of scopolamine causes an anxiety-like behaviour and depressive-like response, in accordance with our previous investigations.\(^{[19,20,54,55]}\) In a
Among these monoterpenes, monoterpene hydrocarbons, including α-pinene (24.10%), β-pinene (24.10%) and β-phellandrene (20.50%), which accounted for 79.40% of the total essential oil, could be responsible for the observed anxiolytic-antidepressant activity as previously reported.

Figure 4. Effects of the inhaled Ferulagoangulata essential oil (FRG1% and FRG3%) on DNA fragmentation in the rat amygdala by agarose (1.5%) gel electrophoresis. Lane 1: DNA ladder; lane 2: control group; lane 3: scopolamine (Sco) alone-treated group; lane 4: Sco+FRG1% group and lane 5: Sco+FRG3% group

In our laboratory, previous studies on different well-known essential oils extracted from Ocimum sanctum L. and Ocimum basilicum L., Coriandrum sativum var. microcarpum, Lavandula angustifolia ssp. angustifolia Mill. and Lavandula hybrida Rev. showed positive effects for a variety of health concerns, including anxiety and depression, in a rat model of Alzheimer’s disease. Using GC-MS/FID analysis, we evidenced that monoterpenols (mainly linalool) were the most important groups of components for these essential oils, used in folk medicine around the world to relieve anxiety and depression. Our high-α-pinene (24.10%) containing F. angulata essential oil could be a good candidate against anxiety and depression in a rat model of Alzheimer’s disease. It has been reported that linalool and β-pinene exhibited antidepressant activity through the monoaminergic pathway in laboratory mice.

Recently, the first three-dimensional structure of a GABA<sub>A</sub> receptor, the human β-3 homopentamer, at 3Å resolution, has been reported. This structure reveals architectural elements unique to eukaryotic Cys-loop receptors, explains the mechanistic consequences of multiple human disease mutations and shows an unexpected structural role for a conserved N-linked glycan. The receptor was crystallized bound to a previously unknown agonist, benzamidine, opening a new avenue for the rational design of GABA<sub>A</sub> receptor modulators. Our study provides the computational results of the interaction between F. angulata essential oil compounds, diazepam, benzamidine and GABA as ligands with GABA<sub>A</sub> protein as a receptor. The interaction energy calculated by AutoDock4 indicates that the receptor of GABA<sub>A</sub> interacts with α-pinene (binding energy -6.00), β-pinene (binding energy -6.00), α-phellandrene (binding energy -5.94) and β-phellandrene (binding energy -5.95) from F. angulata essential oil effectively and among which diazepam (binding energy -7.20) exhibits a close interaction with the GABA<sub>A</sub> receptor. These results attested the anxiolytic profile of the F. angulata essential oil compounds related to anxiolytic effects of diazepam theoretically.

The elevated plus-maze is recognized as a valuable model able to predict the anxiolytic- or anxiogenic-like effects of drugs in rodents.

Our data show that injection of scopolamine significantly decreased the percentage of the time spent in the open arms and the number of open-arm entries in the elevated plus maze test, two indicative parameters of anxiety. This indicates that the scopolamine-treated rats experienced high levels of anxiety and were suitable for evaluating the presumed anxiolytic substances as our essential oil. Furthermore, after the scopolamine-treated rats being exposed to F. angulata essential oil (FRG1% and FRG3%), the percentage of time spent in the open arms significantly increased in the Sco+FRG3% group as compared with scopolamine-alone treated rats. Additionally, the number of open arms entries increased in the Sco+FRG1% group as compared with scopolamine-alone treated rats. However, significant differences were observed between both doses of F. angulata essential oil (FRG1% and FRG3%) on the percentage of time spent in the open arms and on the number of open arms entries in the elevated plus-maze task. These results are strengthened by the fact that the benzodiazepine diazepam (DZP), well-known as positive standard anxiolytic, was used as a positive control comparably to the F. angulata essential oil (FRG1% and FRG3%) in all of our experimental conditions. As expected, DZP produced significant increases in the percentage of time spent in the open arms and the number of open-arm entries as compared with 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. The pharmacological action of diazepam enhances the effect of the neurotransmitter GABA by binding to the benzodiazepine site on the GABA<sub>A</sub> receptor (via the constituent chloride ion) leading to central nervous system (CNS) depression. 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<sub>A</sub> receptor complex. Moreover, it has been reported that β-pinene showed antidepressant-like and sedative-like activity. A previous study reported no difference between the potentiated response of GABA on GABA<sub>A</sub> receptors in the presence of α-pine and β-pinene, suggesting that both have a sedative effect. In light of these reports, our high-α-pine (24.10%) and β-pinene (22.70%) containing <em>F. angulata</em> essential oil have increased the anxiolytic-like behaviour and anti-depressive-like response in scopolamine-treated rats.

The present data suggest that injection of scopolamine significantly decreased the swimming time and increased the immobility time as compared with the control rats, two indicative parameters of depression. This indicates that the scopolamine alone-treated rats exhibited depression. After being exposed to both doses of <em>F. angulata</em> essential oil (FRG1% and FRG3%), the swimming time significantly increased, especially in the Sco+FRG3% group. Additionally, the decrease of the immobility time, especially in the Sco+FRG3% group was also observed. These results suggested that <em>F. angulata</em> essential oil, but especially FRG3%, possesses a strong antidepressant-like response to an inescapable stress. However, significant differences were observed between both doses of <em>F. angulata</em> essential oil (FRG1% and FRG3%) on the swimming time and in the immobility time in the forced swimming test. In our study, tramadol (TRM), as a positive control, produced significant increases in the swimming time and decreases in the immobility time as compared with 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 anergic, and it is also considered as an antidepressant.

Moreover, it is important to note that oxidative stress is believed to be a critical factor in AD. The CNS is very susceptible to oxidative stress as the brain has a high consumption of oxygen, contains large amounts of free-radical generating iron and substances like ascorbate, glutamate and polyunsaturated fatty acids, that easily undergo redox-reaction leading to radicals' formation and exhibits relatively poor antioxidant defense systems. Scopolamine is connected with increased oxidative stress in the whole brain, as well as in particular structures associated with memory and learning.

In our study, we observed a significant decrease in SOD, GPX and CAT-specific activities and the total content of reduced GSH along with elevated protein carbonyl and MDA levels in the amygdala homogenates of the scopolamine alone-treated rats. Protein oxidation is an important factor in aging and age-related neurodegenerative disorders. Protein oxidation is most often indexed by the presence of protein carbonyls which arise from a direct free radical attack on vulnerable amino acids side chains or from the products of glycation, glycoxidation and lipid peroxidation reactions with protein. Lipid peroxidation is one of the major outcomes of free radical mediated injury that directly damages membranes and generates some secondary products including aldehydes, such as MDA. Analysis of AD brains demonstrates an increase in lipid peroxidation products in the amygdala of the AD brain compared with age-matched controls.

Our results imply that <em>F. angulata</em> essential oil (FRG1% and FRG3%), but especially FRG1%, was able to overwhelm the pro-oxidant effects of scopolamine in the rat amygdala homogenates evidenced by an increase in antioxidant enzymes activities (SOD, GPX and CAT) and the total content of reduced GSH and the decrease of protein carbonyl and MDA levels. Also, we reported the absence of DNA cleavage patterns in the amygdala of the scopolamine-treated rats exposed to <em>F. angulata</em> essential oil (FRG1% and FRG3%), suggesting that the essential oil did not induce the apoptotic events.

Conclusions

Taken together, our findings suggest that the <em>Ferulago angulata</em> subsp. <em>carduchorum</em> essential oil has anxiolytic and antidepressant effects and also exhibited antioxidant effects by alleviation of oxidative stress induced by scopolamine in the rat amygdala. In conclusion, inhalation of <em>F. angulata</em> essential oil might offer a useful alternative or complementary choice in either the prevention or the treatment of a psychiatric condition closely related to AD conditions.

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References
