

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

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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_A 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^[1] and constitutes approximately two-thirds of all cases of dementia.^[2] The hallmark pathology of AD comprised of widespread amyloid-beta (A β) deposition, neurofibrillary tangle formation and extensive neurodegeneration in the brain. AD pathology is formed by the complex interaction between multiple genetic and environmental factors.^[3]

Currently, no effective treatments for AD are available.^[4] Progressive cognitive and behavioural impairment is characteristic in AD.^[5] There is evidence indicating that the pathological process begins years if not decades before clinical symptoms occur.^[6]

Although cognitive symptoms are characteristic of AD, non-cognitive symptoms are becoming increasingly important because of the prevalence and dysfunctions they generate.^[7] 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.^[5] These symptoms increase the caregiver stress and impairment in daily living activities, worsen the patient's quality of life and also accelerate cognitive decline.^[8]

The amygdala is one of the early structures to undergo neurodegeneration in AD.^[9] 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.^[10] It has been shown that substantial atrophy within the amygdala in

AD was evidenced.^[11] It is associated with a role in emotional processing and the storage of emotional memories.^[11] The amygdala is the key structure in the acquisition and expression of fear,^[12] influencing affective states such as depression and anxiety.^[9] It is known that amygdala lesions have deleterious consequences on primate social behaviour.^[13]

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).^[14] A decline in GABA_A receptor signalling triggers hyperactive neurological disorders such as insomnia, anxiety and epilepsy. In response to binding the neurotransmitter GABA, released at inhibitory synapses, GABA_A receptor chloride channels open and depress neuronal excitability in the adult central nervous system.^[15] 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.^[16] 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.^[17]

Scopolamine is a blocker of the muscarinic acetylcholine receptor that serves as a beneficial pharmacological tool in producing a model of amnesia.^[18,19] Scopolamine is used as a standard/reference drug for inducing cognitive deficits in healthy humans and animals.^[20] 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.^[21] Inhibition of the muscarinic acetylcholine receptor by scopolamine also contributes to characteristic cognitive and memory deficits of AD,^[22] as well as the cholinergic receptor antagonists.^[23] There is notable evidence that scopolamine causes oxidative stress through the interference with acetylcholine in the brain leading to cognitive impairment.^[18]

Ferulago angulata L. is widespread in the high altitudes of several Asian countries such as Iraq and Iran as well as Turkey.^[24,25] The genus *Ferulago* is represented by 50 species worldwide and 31 of which are found in Turkey.^[26] *Ferulago angulata* was divided into two subspecies by Chamberlain in 1987. The known subspecies distributed in Turkey is *F. angulata* subsp. *angulata*; subsp. *carduchorum* differs from subsp. *angulata* by having scabrid inflorescence, ovary and leaves (not glabrous or subglabrous).^[26]

In folk medicine, different species of *Ferulago* has been used in Turkey and Iran as sedative, tonic and remedy of digestive panics, aphoristic properties and haemorrhoids. Moreover, different parts of *Ferulago* species have been traditionally used against ulcers, snake bite and for the treatment of a headache and disease of the spleen.^[27,28] The essential oils of *F. campestris* were determined to have antioxidant and antifungal effects.^[29] The essential oils of *F. sandrasica* were also reported to have antibacterial and antioxidant activities with the emphasis that phenolic compounds may be responsible for the antioxidant activity.^[30] Moreover, *F. angulata* essential oil was determined to have antioxidant activities.^[31] In addition, it has been reported that *Ferula assafoetida* L. exhibited anxiolytic effects in rodents within the elevated plus-maze model of anxiety.^[32] To our knowledge, this is the first report on the effects of *F. angulata* subsp. *carduchorum* essential oil on anxiety and depression responses of a scopolamine-induced rat model of AD. Here we hypothesized that exposure to *F. angulata* essential oil could ameliorate scopolamine-induced anxiety and the depression effects and also decrease the oxidative stress in the rat amygdala.

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 Bingöl, Eastern Anatolia, Turkey, in June 2013 and identified by Dr Eyup Bağcı 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.^[33]

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, Elazığ, Turkey. This study was approved by the Committee on the Ethics of Laboratory Animal Experiments of the Firat University, Elazığ, 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 Plexiglas 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. *Ferulago angulata* essential oil was diluted with 1% Tween 80 (v/v). *Ferulago 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

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^[34] from RCSB PDB^[35] was used. The molecular docking procedure was carried out using VEGA ZZ, a complete molecular modelling suite^[36] in conjunction with NAMD^[37] and AutoDock4.^[38]

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.^[39] 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_A receptor has five neurotransmitter-binding sites.^[34] To generate complexes in which the ligand is placed in the same pocket of the co-crystallized one, the atoms selected included a 10 Å 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 (CID 119), diazepam (CID 3016) and benzamidine (CID 2332). The structures of the compounds were provided by PubChem Compound Database.^[40] For the docking procedure, VEGA ZZ was used in conjunction with GriDock/AutoDock4 and the receptor structure was pre-processed eliminating the water molecules, assigning the AMBER atom types, fixing the atom charges, removing the apolar hydrogens and saving the molecule in a PDBQT format. The images for the docking results were processed with PyMOL and POV-Ray.

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.^[41] The percentages of time spent in the open arms (time spent in the open arms/time spent in all arms $\times$ 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.^[42] The depressive-like response was assessed, basically using the same method described by Campos *et al.*,^[43] 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 μ M riboflavin, 6 mM EDTA and 200 μ l 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.*^[44] 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.^[45] The reaction mixture consisted of 150 μ l phosphate buffer (0.01 M, pH 7.0), 100 μ l supernatants. The reaction was started by adding 250 μ l 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 μ mol of H₂O₂ consumed/min/mg protein.

Determination of amygdala GPX activity

Glutathione peroxidase (GPX, E.C. 1.11.1.9) activity was analysed 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 Na₂S₂O₃, 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 Oliver *et al.*^[48] and according to the indications of Luo and Wehr.^[49] 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 at 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 at 4 °C. The absorbance at 370 nm of the DNPH-treated sample versus the HCl control was recorded, and the results are expressed as nmols of DNPH incorporated/mg of protein based on an average absorptivity of 21 mM⁻¹ cm⁻¹ for most aliphatic hydrazones.

Determination of the amygdala MDA level

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 20 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.

Estimation of protein concentration

Estimation of protein was done using a bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich). The BCA protein assay is a detergent-compatible formulation based on BCA for the

colorimetric detection and quantification of total protein, as previously described by Smith *et al.*^[51]

DNA fragmentation

Total DNA was isolated from the amygdala samples using the phenol/chloroform method as previously described by Ausubel *et al.*^[52] In total, 50 mg of tissue sample was digested overnight at 37 °C in 0.6 ml of digestion buffer (100 mM NaCl, 10 mM TRIS/HCl, 25 mM EDTA pH 8.0, 0.5% SDS) containing 0.1 mg/ml proteinase K (Boehringer Mannheim, Germany). The digest was extracted with equal volumes of TRIS-saturated phenol (pH 8.0) (Roti-phenol, Roth, Germany) by shaking gently to mix completely the two phases. The phases were then separated by centrifugation, and the aqueous phase (approx. 0.6 ml) was transferred to another tube avoiding interphase. The DNA was then precipitated by adding 300 µl of 7.5 M ammonium acetate (i.e. 1/2 of volume) and an equal volume of 100% ethanol at room temperature and shaken gently to mix thoroughly. DNA seen as a stringy precipitate was pelleted by centrifugation and washed with 70% ethanol to remove traces of sodium dodecyl sulfate and phenol. After removing the ethanol, the DNA was air-dried for 10 min at room temperature and suspended with 50 µl of 10 mM TRIS (pH 8.0), 1 mM EDTA. The DNA content was determined spectrophotometrically by absorbance at 260 nm and the purity of the DNA was confirmed by a ratio > 1.8 at 260/280 nm. Approximately 0.5 mg of genomic DNA was dissolved in a mixture of 10 µl of TRIS-EDTA and 5 µl of gel loading buffer [0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% (v/v) glycerol] and then loaded on a 1.5% agarose gel in TRIS-boric acid-EDTA (TBE) buffer (89 mM Tris-boric acid, 2 mM EDTA, pH 8.0). Electrophoresis was performed in TBE at 120 V until a sufficient resolution was obtained. A 1-kb DNA ladder (New England Biolabs, Ipswich, MA, USA) was used as a standard size marker. The bands were visualized by ethidium bromide staining under UV light.

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 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.

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

No.	Compounds	RI ^a	RI ^b	Concentration (%)
1.	heptanal	885	850	0.10
2.	α -thujene	930	928	0.20
3.	α -pinene	939	937	24.10
4.	camphene	953	950	0.30
5.	sabinene	976	975	1.50
6.	β -pinene	980	977	22.70
7.	β -myrcene	991	989	2.40
8.	α -phellandrene	1005	1003	12.10
9.	α -terpinene	1018	1013	0.10
10.	p-cymene	1026	1018	3.20
11.	β -phellandrene	1006	1002	20.50
12.	β -ocimene	1056	1054	0.50
13.	γ -terpinene	1062	1059	0.30
14.	p-cresol	2094	2073	0.10
15.	α -terpinolene	1088	1087	0.70
16.	benzene, 1-methyl-4-(1-methylethenyl)-	1131	1139	0.10
17.	bicyclohexyl-2-one	1170	1166	0.10
18.	trans-pinocarveol	1135	1142	0.20
19.	2,5-diethylthiophene	1196	1194	1.70
20.	3-cyclohexen-1-ol	1177	1204	0.30
21.	1,3-cyclohexadiene	1272	1245	0.60
22.	α -terpineol	1186	1193	0.10
23.	thymol	1232	1274	0.50
24.	carvacrol	1250	1241	0.20
25.	α -copaene	1376	1360	0.10
26.	β -elemene	1375	1370	0.20
27.	caryophyllene	1582	1581	0.10
28.	γ -elemene	1430	1397	0.10
29.	β -cubebene	1387	1388	0.10
30.	trans- β -farnesene	1505	1512	0.10
31.	α -humulene	1459	1451	0.10
32.	aromadendrene	1449	1441	0.40
33.	trans- β -lanone	1488	1433	0.10
34.	germacrene D	1480	1479	0.40
35.	bicylogermacrene	1501	1448	0.10
36.	α -selinene	1498	1492	0.10
37.	α -amorphene	1474	1456	0.10
38.	δ -cadinene	1524	1523	0.10
39.	cis- α -bisabolene	1496	1470	0.10
40.	germacren B	1560	1561	0.20
41.	trans- β -caryophyllene	1418	1416	0.10
42.	tau-murolol	1644	1649	0.30
43.	α -cadinol	1652	1657	0.10
44.	β -humulene	1448	1442	0.10
45.	α -bisabolol	1672	1681	0.60
46.	dehydroaromadendrene	1461	1457	0.10
47.	benzyl benzoate	1759	1760	0.10
48.	tricosane	2300	2300	0.10
	Total			96.50

RI^a, literature retention indices.
RI^a, experimental retention indices relative to *n*-alkanes on the HP-5 MS column.

Docking studies of receptor-ligand interactions

The GABA_A receptor- $\beta 3_{\text{cryst}}$ is a homopentamer and has five similar conformation neurotransmitter binding sites located between the extracellular domains.^[34] 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_A receptor, the ligand molecules were docked with the target protein using VEGA ZZ in conjunction with GriDock/AutoDock4. Every docking contains some useful knowledge that includes the inhibition constant (k_i), 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_A, gave the best docking results. According to Table 2, these compounds present the smaller value of k_i 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_A 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 ($P < 0.001$), the Sco vs. the Sco+FRG3% groups ($P < 0.01$) and the Sco+FRG1% and the Sco+FRG3% groups ($P < 0.001$) 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 ($P < 0.0001$), 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 ($P < 0.01$) for the swimming time

Table 2. Ligand–protein interaction parameters

Molecule	Inhibition constant, K_i (μ M)	Free energy of binding (kcal/mol)	Total inter-molecular energy (kcal/mol)	Final total internal energy (kcal/mol)	Torsional free energy (kcal/mol)	Unbound system's energy (kcal/mol)	PubChem CID
benzamidine	313.00	-4.78	-5.38	-0.04	0.60	-0.04	2332
benzamidine-4COF	1520.00	-3.85	-4.78	0.04	0.89	0.00	-
diazepam	5.24	-7.20	-7.7	-0.43	0.30	-0.40	3016
GABA	3460.00	-3.36	-4.26	-0.01	0.89	-0.02	119
α -pinene	40.30	-6.00	-6.00	0.00	0.00	0.00	6654
β -pinene	40.00	-6.00	-6.00	0.00	0.00	0.00	14896
α -phellandrene	44.50	-5.94	-5.94	0.00	0.00	0.00	7460
β -phellandrene	43.00	-5.95	-5.95	0.00	0.00	0.00	7460
2,5-diethylthiophene	179.00	-5.11	-5.71	-0.09	0.60	-0.09	521294
β -myrcene	269.00	-4.87	-6.02	-0.20	1.19	-0.16	31253
p-cymene	58.40	-5.78	-5.78	0.00	0.00	0.00	7463
sabinene	29.20	-6.19	-6.19	0.00	0.00	0.00	18818

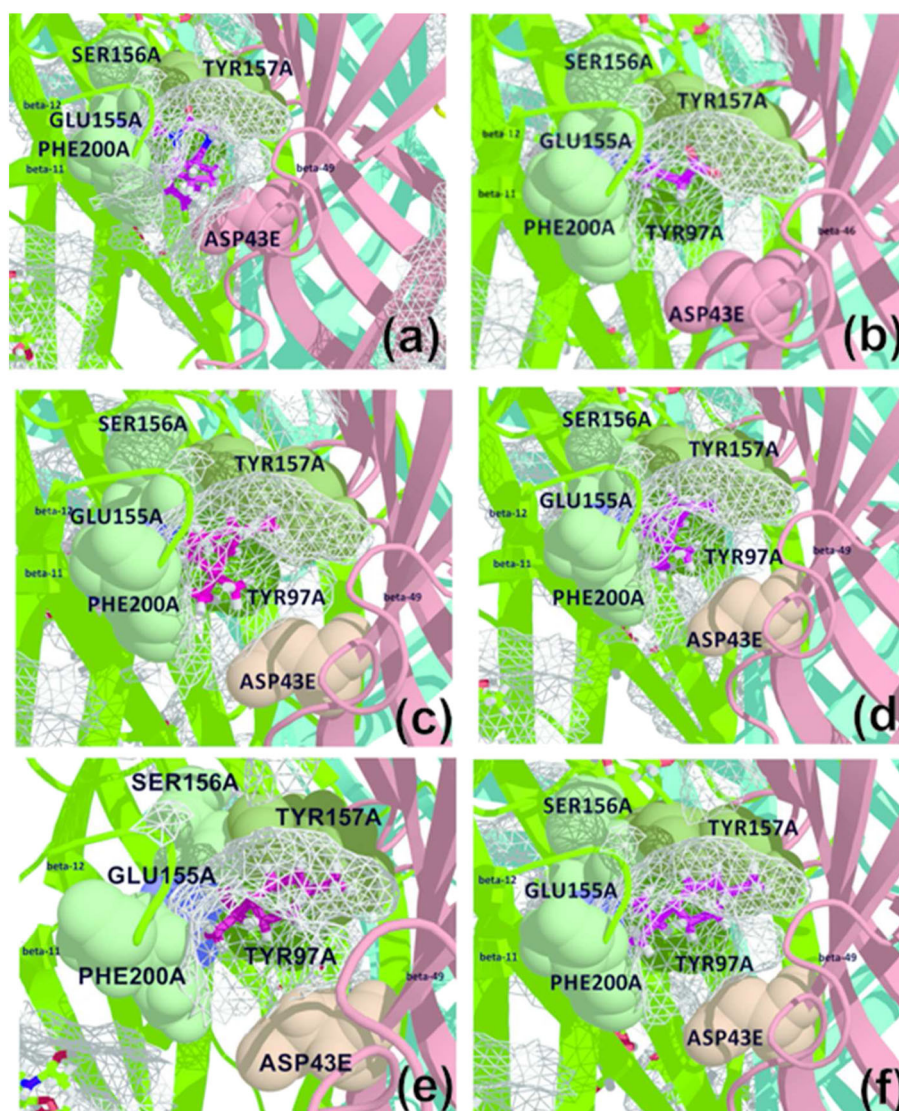


Figure 1. The binding surface and the docking position for molecule (a) diazepam (CID 3016), (b) GABA (CID 119), (c) α -pinene (CID 6654), (d) β -pinene (CID 14896), (e) α -phellandrene (CID 7460) and β -phellandrene (CID 11142) as depicted in magenta

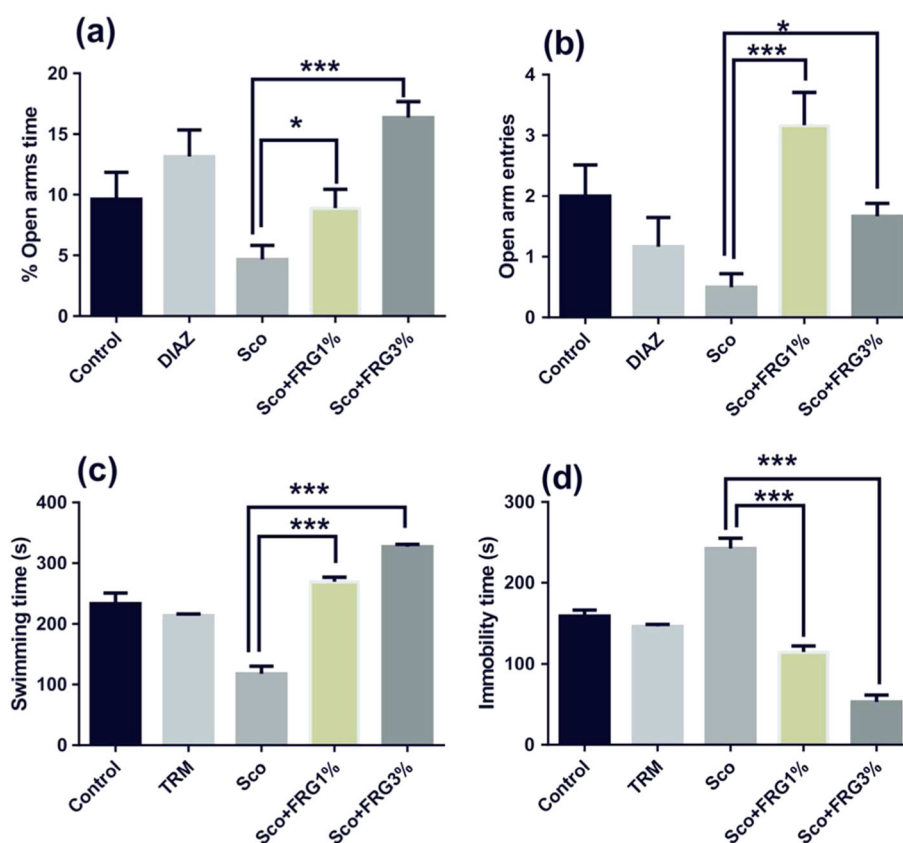


Figure 2. Effects of the inhaled *Ferulago angulata* essential oil (FRG1% and FRG3%) in the elevated plus-maze test on the percentage of the time spent in the open arms (a) and on the number of open-arm entries (b) and in the forced swimming test on swimming time (c) and on the immobility time (d) in the scopolamine (Sco)-treated rats. Values are the means $\pm$ SEM ($n = 6$ animals per group), * $P < 0.001$ and *** $P < 0.0001$ vs. the scopolamine (Sco) alone-treated group

(Figure 2c). 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 ($P < 0.01$), 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).

Effect of the *F. angulata* essential oil on SOD, GPX and CAT activities

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).

Effect of the *F. angulata* essential oil on the total content of reduced GSH, protein carbonyl and MDA levels

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).

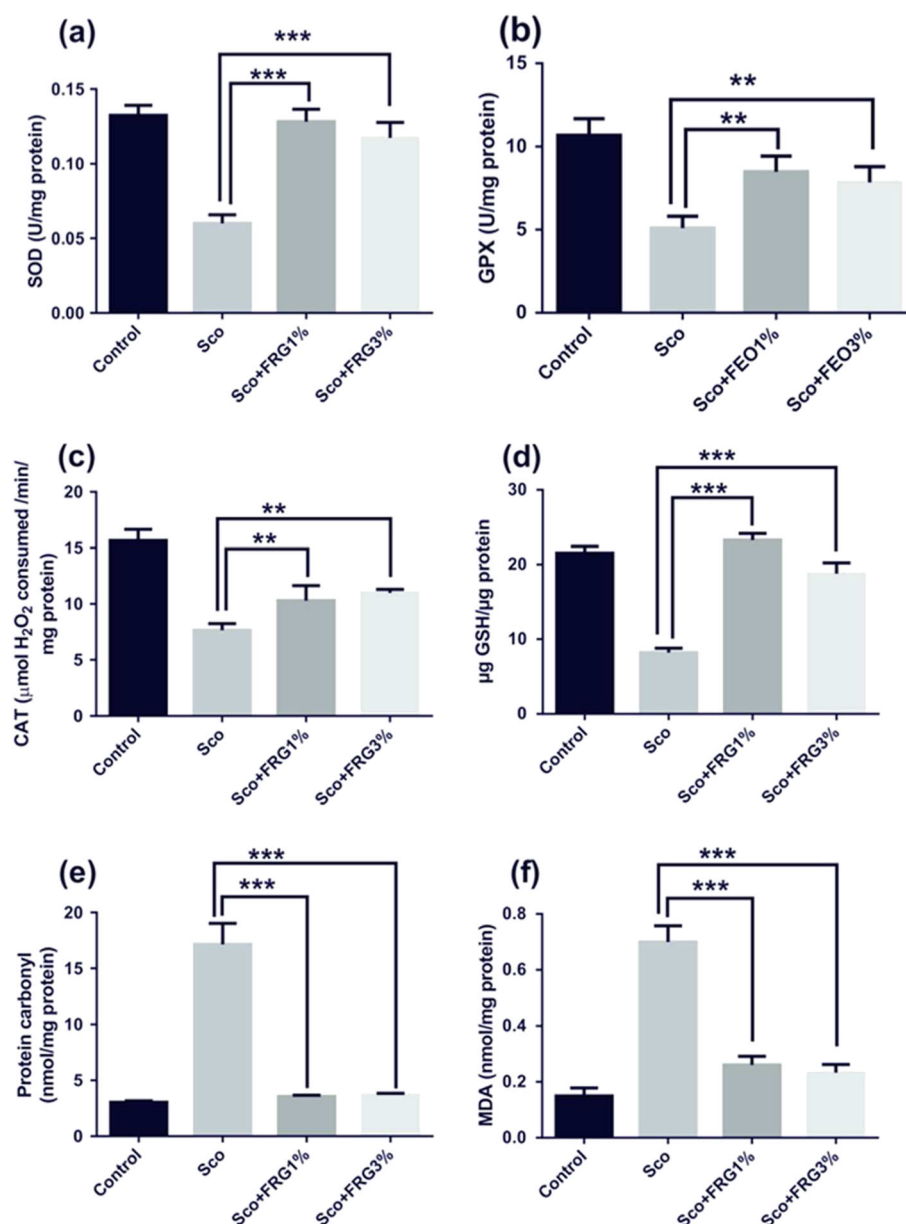


Figure 3. Effects of the inhaled *Ferulago angulata* essential oil (FRG1% and FRG3%) on SOD (a), GPX (b) and CAT (c)-specific activities, on the total content of reduced GSH (d), protein carbonyl (e) and MDA (f) levels in the scopolamine (Sco)-treated rats. Values are the means $\pm$ SEM ($n = 6$ animals per group), ** $P < 0.01$ and *** $P < 0.0001$ vs. the scopolamine (Sco) alone-treated group

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 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 μ l, 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

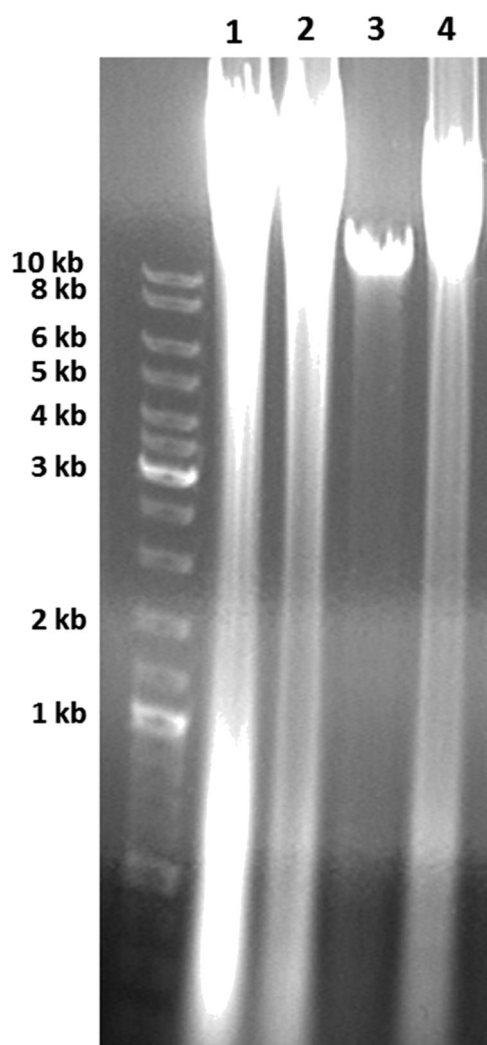


Figure 4. Effects of the inhaled *Ferulago angulata* essential oil (FRG1% and FRG3%) on DNA fragmentation in the rat amygdala by agarose (1.5%) gel electrophoresis. Lane 1: DNA ladder; lane 2: control group; lane 3: scopolamine (Sco) alone-treated group; lane 4: Sco+FRG1% group and lane 5: Sco+FRG3%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

study that assessed light-dark preferences, it was reported that scopolamine (2 mg/kg, i.p.) decreased the number of transitions to the light side.^[54] Also, in the black-white box, scopolamine (0.05, 0.1 mg/kg, i.p.) was found to increase anxiety-related behaviour on both activity dependent as well as activity-independent parameters.^[55]

The GC-MS/FID analysis of our *F. angulata* essential oil indicated monoterpene hydrocarbons, 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, as the main components of the essential oil suggesting that these constituents could be responsible for the observed anxiolytic-antidepressant-like behaviour in scopolamine-treated rats. Among these monoterpenes, α -pinene (24.10%) was identified in high amounts and it has been reported to present anxiolytic effects.^[56–58] The monoterpene β -pinene (22.70%) identified in our essential oil as the main active compound showed antidepressant-like and sedative-like activity as previously reported.^[59]

In our laboratory, previous studies on different well-known essential oils extracted from *Ocimum sanctum* L. and *Ocimum basilicum* L.,^[60] *Coriandrum sativum* var. *microcarpum*,^[61] *Lavandula angustifolia* ssp. *angustifolia* Mill. and *Lavandula hybrida* Rev.^[19] 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%) and β -pinene (22.70%) 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.^[62]

Recently, the first three-dimensional structure of a GABA_A receptor, the human β -3 homopentamer, at 3 Å 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_A receptor modulators.^[34] Our study provides the computational results of the interaction between *F. angulata* essential oil compounds, diazepam, benzamidine and GABA as ligands with GABA_A protein as a receptor. The interaction energy calculated by AutoDock4 indicates that the receptor of GABA_A 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_A 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.^[63]

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.^[41] 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,^[64] was used as a positive control comparatively 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.^[65,66] 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.^[67] 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.^[68] Moreover, it has been reported that β -pinene showed antidepressant-like and sedative-like activity.^[59] A previous study reported no difference between the potentiated response of GABA on GABA_A receptors in the presence of α -pinene and β -pinene,^[69] suggesting that both have a sedative effect. In light of these reports, our high- α -pinene (24.10%) and β -pinene (22.70%) containing *F. angulata* 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.^[70] This indicates that the scopolamine alone-treated rats exhibited depression. After being exposed to both doses of *F. angulata* 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 *F. angulata* essential oil, but especially FRG3%, possesses a strong antidepressant-like response to an inescapable stress. However, significant differences were observed between both doses of *F. angulata* 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 analgesic, and it is also considered as an antidepressant.^[71]

Moreover, it is important to note that oxidative stress is believed to be a critical factor in AD.^[72] 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.^[73] Scopolamine is connected with increased oxidative stress in the whole brain, as well as in particular structures associated with memory and learning.^[74]

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.^[75,76] Protein oxidation is most often indexed by the presence of protein carbonyls,^[76] 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.^[77] 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.^[78] Analysis of AD brains demonstrates an increase in lipid peroxidation products in the amygdala of the AD brain compared with age-matched controls.^[79]

Our results imply that *F. angulata* 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 *F. angulata* essential oil (FRG1% and FRG3%), suggesting that the essential oil did not induce the apoptotic events.

Conclusions

Taken together, our findings suggest that the *Ferulago angulata* subsp. *carduchorum* 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 *F. angulata* 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

1. A. Samadi, M. Estrada, C. Pérez, M. I. Rodríguez-Franco, I. Iriepa, I. Moraleda, M. Chioua, J. Marco-Contelles. *Eur. J. Med. Chem.* **2012**, *57*, 296.
2. Q. Wang, B. Xiao, S. Cui, H. Song, Y. Qian, L. Dong, H. An, Y. Cui, W. Zhang, Y. He, J. Zhang, J. Yang, F. Zhang, G. Hu, X. Gong, Z. Yan, Y. Zheng, X. Wang. *DMM* **2014**, *7*, 1385.
3. J.-i. Satoh, Y. Kino, Y. Yamamoto, N. Kawana, T. Ishida, Y. Saito, K. Arima. *Alzheimers Res. Ther.* **2014**, *6*, 1.
4. E. Kontseikova, N. Zilka, B. Kovacech, R. Skrabana, M. Novak. *Alzheimers Res. Ther.* **2014**, *6*, 45.
5. S. Takeda, N. Sato, R. Morishita. *Front Aging Neurosci.* **2014**, *6*, 171.
6. T. Arendt, J. Stieler, M. Holzer. *J. Neural Transm.* **2014**, *1*.
7. T. Cañete, G. Blázquez, A. Tobeña, L. Gimenez-Llort, A. Fernández-Teruel. *Behav. Brain Res.* **2015**, *281*, 156.
8. M. Fernandez, A. Gobartt, M. Balana, t. C. S. Group. *BMC Neurol.* **2010**, *10*, 87.
9. R. C. Klein, S. K. Acheson, B. E. Mace, P. M. Sullivan, S. D. Moore. *Neurobiol. Aging* **2014**, *35*, 2046.
10. E. Cavedo, M. Boccardi, R. Ganzola, E. Canu, A. Beltramello, C. Caltagirone, P. M. Thompson, G. B. Frisoni. *Neurology* **2011**, *76*, 727.
11. M. I. Miller, L. Younes, J. T. Ratnanather, T. Brown, H. Trinh, D. S. Lee, D. Tward, P. B. Mahon, S. Mori, M. Albert. *Neurobiol. Aging* **2015**, *36*(Supplement 1), S3.
12. D. Schiller, I. Levy, Y. Niv, J. E. LeDoux, E. A. Phelps. *J. Neurosci.* **2008**, *28*, 11517.
13. S. G. Costafreda, M. J. Brammer, A. S. David, C. H. Y. Fu. *Brain Res. Rev.* **2008**, *58*, 57.
14. A. Skórzewska, M. Lehner, A. Wiśłowska-Stanek, D. Turzyńska, A. Sobolewska, P. Krząciak, A. Płaźnik. *Pharmacol. Biochem. Behav.* **2015**, *129*, 34.
15. L. E. Rabow, S. J. Russek, D. H. Farb. *Synapse* **1995**, *21*, 189.
16. S. Subash, N. Braid, M. M. Essa, A.-B. Zayana, V. Ragini, S. Al-Adawi, A. Al-Asmi, G. J. Guillemin. *Nutrition* **2015**, *31*, 223.
17. M. Dumont, M. F. Beal. *Free Radic. Biol. Med.* **2011**, *51*, 1014.

18. S. Rahnama, Z. Rabiei, Z. Alibabaei, S. Mokhtari, M. Rafieian-kopaei, F. Deris. *Neurol. Sci.* **2015**, 36, 553.
19. L. Hritcu, O. Cioanca, M. Hancianu. *Phytomedicine* **2012**, 19, 529.
20. I. Klinkenberg, A. Blokland. *Neurosci. Biobehav. R* **2010**, 34, 1307.
21. W. Chen, X. Cheng, J. Chen, X. Yi, D. Nie, X. Sun, J. Qin, M. Tian, G. Jin, X. Zhang. *PLoS One* **2014**, 9, e88076.
22. D. Y. Yoo, W. Kim, K.-Y. Yoo, C. H. Lee, J. H. Choi, I.-J. Kang, Y. S. Yoon, D.-W. Kim, M.-H. Won, I. K. Hwang. *Phytother. Res.* **2011**, 25, 809.
23. S. Kotani, T. Yamauchi, T. Teramoto, H. Ogura. *Chem.-Biol. Interact.* **2008**, 175, 227.
24. M. Rafieian-Kopaei, N. Shahinfard, H. Rouhi-Boroujeni, M. Gharipour, P. Darvishzadeh-Boroujeni. *Evid. Based Compl. Alternat. Med.* **2014**, 2014, 680856.
25. H. R. Ghasempour, E. Shirinpour, H. Heidari. *IJST* **2007**, 31, 309.
26. L. Behçet, I. Kaval, M. Rüstemoğlu. *Turk. J. Bot.* **2012**, 36, 637.
27. B. Ozturk, S. Gur, M. Coskun, M. Kusan, C. Erdurak, C. Hafez, G. Ozgunes, M. Cetinkaya. *Eur. Urol. Suppl.* **2004**, 3, 62.
28. G. Sodefian, K. Ansari, A. Bamoniri, B. Mirjalili. *Dig. J. Nonomat. Biostruct.* **2011**, 6, 162.
29. C. Cecchini, M. M. Coman, A. Cresci, B. Tirillini, G. Cristalli, F. Papa, G. Sagratini, S. Vittori, F. Maggi. *Flavour Frag. J.* **2010**, 25, 493.
30. A. Celik, I. Arslan, E. N. Herken, A. Ermis. *Int. J. Food Prop.* **2013**, 16, 1665.
31. A. Pirbalouti, L. Sedaghat, B. Hamed, F. Tirgir. *Bangladesh J. Bot.* **2014**, 42, 327.
32. A. Alqasoumi. *J. Pharmacognosy Phytother.* **2012**, 4, 86.
33. R. P. Adams. *Identification of essential oil by Gas Chromatography/Mass Spectroscopy*. Allured Publishing Corporation: Carol Stream, Illinois, **2007**.
34. P. S. Miller, A. R. Aricescu. *Nature* **2014**, 512, 270.
35. H. M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, I. N. Shindyalov, P. E. Bourne. *Nucleic Acids Res.* **2000**, 28, 235.
36. A. Pedretti, L. Villa, G. Vistoli. *J. Comput. Aided Mol. Des.* **2004**, 18, 167.
37. J. C. Phillips, R. Braun, W. Wang, J. Gumbart, E. Tajkhorshid, E. Villa, C. Chipot, R. D. Skeel, L. Kale, K. Schulten. *J. Comput. Chem.* **2005**, 26, 1781.
38. G. M. Morris, R. Huey, W. Lindstrom, M. F. Sanner, R. K. Belew, D. S. Goodsell, A. J. Olson. *J. Comput. Chem.* **2009**, 16, 2785.
39. P. Labute. <http://www.chemcomp.com/journal/proton.htm> **2007**.
40. E. Bolton, Y. Wang, P. A. Thiessen, S. H. Bryant. *Annu. Rep. Comput. Chem.* **2008**, 4, 217.
41. Y. Hayashi, S. Sogabe, Y. Hattori, J. Tanaka. *Neurosci. Lett.* **2012**, 531, 166.
42. J. F. Cryan, A. Markou, I. Lucki. *Trends Pharmacol. Sci.* **2002**, 23, 238.
43. M. Campos, E. Fernandes, J. Ferreira, A. S. Santos, J. Calixto. *Psychopharmacol (Berl)* **2005**, 182, 45.
44. C. Winterbourn, R. Hawkins, M. Brian, R. Carrell. *J. Lab. Clin. Med.* **1975**, 85, 337.
45. A. K. Sinha. *Anal. Biochem.* **1972**, 47, 389.
46. M. Sharma, Y. K. Gupta. *Life Sci.* **2002**, 7, 2489.
47. K. Fukuzawa, A. Tokumura. *J. Nutr. Sci. Vitaminol.* **1976**, 22, 405.
48. C. N. Oliver, B. W. Ahn, E. J. Moerman, S. Goldstein, E. R. Stadtman. *J. Biol. Chem.* **1987**, 262, 5488.
49. S. Luo, N. B. Wehr. *Redox Rep.* **2009**, 14, 159.
50. H. Ohkawa, N. Ohishi, K. Yagi. *Anal. Biochem.* **1979**, 95, 351.
51. P. K. Smith, R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, D. C. Klenk. *Anal. Biochem.* **1985**, 150, 76.
52. F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, K. Struhl. *Current Protocol in Molecular Biology*. Wiley-Interscience: New York, **2002**.
53. V. d. M. Linck, A. L. da Silva, M. Figueiró, Â. Luis Piato, A. Paula Herrmann, F. Dupont Birk, E. Bastos Caramão, D. Sávio Nunes, P. R. H. Moreno, E. Elisabetsky. *Phytomedicine* **2009**, 16, 303.
54. R. N. Hughes, C. S. Desmond, L. C. E. Fisher. *Behav. Processes* **2004**, 67, 173.
55. J. W. Smythe, D. Murphy, S. Bhatnagar, C. Timothy, B. Costall. *Pharmacol. Biochem. Behav.* **1996**, 54, 57.
56. T. Satou, H. Kasuya, K. Maeda, K. Koike. *Phytother. Res.* **2014**, 28, 1284.
57. T. Satou, S. Murakami, M. Matsuura, S. Hayashi, K. Koike. *Nat. Prod. Commun.* **2010**, 5, 143.
58. H. Kasuya, E. Hata, T. Satou, M. Yoshikawa, S. Hayashi, Y. Masuo, K. Koike. *Nat. Prod. Commun.* **2013**, 8, 515.
59. S. L. Guzmán-Gutiérrez, R. Gómez-Cansino, J. C. García-Zebadúa, N. C. Jiménez-Pérez, R. Reyes-Chilpa. *J. Ethnopharmacol.* **2012**, 143, 673.
60. V. Gradinaru, O. Cioanca, L. Hritcu, A. Trifan, E. Gille, M. Hancianu. *Phytochemistry Rev.* **2015**, 14, 567.
61. O. Cioanca, L. Hritcu, M. Mihasan, A. Trifan, M. Hancianu. *Physiol. Behav.* **2014**, 131, 68.
62. S. L. Guzmán-Gutiérrez, H. Bonilla-Jaime, R. Gómez-Cansino, R. Reyes-Chilpa. *Life Sci.* **2015**, 128, 24.
63. A. Blainski, V. K. Piccolo, J. C. P. Mello, R. M. W. de Oliveira. *J. Ethnopharmacol.* **2010**, 128, 541.
64. M. T. Mansouri, M. Soltani, B. Naghizadeh, Y. Farbood, A. Mashak, A. Sarkaki. *Pharmacol. Biochem. Behav.* **2014**, 117, 40.
65. G. Marco Leggio, S. Alfio Torrisi, A. Castorina, C. B. M. Platania, A. A. R. Impellizzeri, A. Fidilio, F. Caraci, C. Bucolo, F. Drago, S. Salomone. *Eur. Neuropsychopharmacol.*, 10.1016/j.euroneuro.2014.11.004.
66. I. F. Adebisin, A. J. Akindele, O. O. Adeyemi. *J. Ethnopharmacol.* **2015**, 160, 101.
67. J. Riss, J. Cloyd, J. Gates, S. Collins. *Acta Neurol. Scand.* **2008**, 118, 69.
68. M. Emamghoreishi, M. Khasaki, M. F. Aazam. *J. Ethnopharmacol.* **2005**, 96, 365.
69. H. Aoshima, K. Hamamoto. *Biosci. Biotechnol. Biochem.* **1999**, 63, 743.
70. A. Bonito-Oliva, D. Masini, G. Fisone. *Front Behav. Neurosci.* **2014**, 8, 290.
71. O. Caspani, M.-C. Reitz, A. Ceci, A. Kremer, R.-D. Treede. *Pharmacol. Biochem. Behav.* **2014**, 124, 290.
72. B. Budzynska, A. Boguszevska-Czubara, M. Kruk-Slomka, K. Skalicka-Wozniak, A. Michalak, I. Musik, G. Biala. *Psychopharmacol (Berl)* **2014**, 1.
73. N. M. Walton, R. Shin, K. Tajinda, C. L. Heusner, J. H. Kogan, S. Miyake, Q. Chen, K. Tamura, M. Matsumoto. *PLoS One* **2012**, 7, e35264.
74. S. D. Pachauri, S. Tota, K. Khandelwal, P. R. P. Verma, C. Nath, K. Hanif, R. Shukla, J. K. Saxena, A. K. Dwivedi. *J. Ethnopharmacol.* **2012**, 139, 34.
75. R. Liu, I. Y. Liu, X. Bi, R. F. Thompson, S. R. Doctrow, B. Malfroy, M. Baudry. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, 100, 8526.
76. E. Stadtman. *Science* **1992**, 257, 1220.
77. J. H. Jhoo, H.-C. Kim, T. Nabeshima, K. Yamada, E.-J. Shin, W.-K. Jhoo, W. Kim, K.-S. Kang, S. A. Jo, J. I. Woo. *Behav. Brain Res.* **2004**, 155, 185.
78. P. Lu, T. Mamiya, L. L. Lu, A. Mouri, L. B. Zou, T. Nagai, M. Hiramatsu, T. Ikejima, T. Nabeshima. *Br. J. Pharmacol.* **2009**, 157, 1270.
79. W. R. Markesbery, M. A. Lovell. *Neurobiol. Aging* **1998**, 19, 33.