Journal of Research in Pharmacy

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The *in vitro* anxiolytic potential of standardized *Lavandula angustifolia* oil and *Humulus lupulus* extract combinations

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Received: 6 April 2026 / Revised: 30 June 2026 / Accepted: 4 July 2026

ABSTRACT: Recent years, particularly following the COVID-19 pandemic, a significant rise in fatigue, depression, and nervous system disorders was observed. While conventional pharmacotherapy is the standard for neuropsychiatric conditions, the use of herbal preparations is gaining prominence. Within the scope of this study, the potential anxiolytic effects of combined standardized herbal preparations were evaluated using the *in vitro* monoamine oxidase (MAO) inhibition assays. The essential oil of *Lavandula angustifolia* Mill. was analyzed and verified by GC/MS, where linalol (22.1%) and linalyl acetate (19.2%) was in compliance with European Pharmacopoeia. Whereas the *Humulus lupulus* L. flower aqueous ethanol extract contained 0.9% humulone according to the HPLC analyses. *In vitro* enzymatic assays showed that the *H. lupulus* extract inhibited MAO-A and MAO-B with IC_{50} values of 47.7 and 50 µg/mL, respectively. Where the *L. angustifolia* essential oil showed IC_{50} 69.4 and 55.1 µg/mL inhibitions, respectively. Using the checkerboard method, the combination of *H. lupulus* (1:4 ratio) and *L. angustifolia* (1:3 ratio) showed 95.5% inhibition of MAO-A, suggesting a syergistic effect, to the best of our knowledge for the first time. These initial experiements suggest that the combinations of lavender oil and hop strobile preparations may be utilized with the potential for development of standardized nervous system targetted phytotherapeutics.

KEYWORDS: Lavender; Hops; MAO inhibition; synergistic combination.

1. INTRODUCTION

Depression is the most prevalent psychiatric disorder globally. While global prevalence rates are estimated between 13% and 20%, the rate within the Turkish population is approximately 10%. Statistical evidence suggests that one in five individuals will experience depressive symptoms at least once in their lifetime. Epidemiological data indicate a higher susceptibility in women (10-25%) compared to men (5-12%), with a peak incidence observed in females aged 25-44 [1]. Furthermore, the COVID-19 pandemic has significantly exacerbated the global prevalence of anxiety and depression. Recent cross-sectional evidence indicates heightened rates of depressive, anxious, and post-traumatic stress disorder (PTSD) symptoms among hospitalized COVID-19 patients. Preliminary data suggest that approximately 20% of these patients develop clinically significant PTSD symptoms within one month of discharge; this highlights the importance of integrated neuropsychiatric care in the post-pandemic era [2,3].

Lavandula species, of the Lamiaceae family contain essential oils, which are particularly widespread in the Mediterranean Region. Lavender is an important medicinal, cosmetic, and perfume rescource, which is cultivated due to its high yield and quality essential oil. The essential oil yield obtained from fresh lavender flowers varies between 1-3% [4]. Linalool and linalyl acetate, which are important essential oil components affecting the quality of lavender oil, are important components [5]. *Lavandula angustifolia* Mill. essential oil and preparations are used for the sedative effects [6-8]. In addition, a study conducted on elderly patients with dementia reported improvement in agitation levels in the patient group who inhaled Lavandula essential oil [9]. According to the European Medicines Agency, *L. angustifolia* essential oil is approved as a traditional herbal medicinal product for the relief of mild symptoms of mental stress, exhaustion, and to aid sleep [10].

How to cite this article: Demirci F, Kahya SE. Anxioloytic potential oil of standardized *Lavandula angustifolia* and *Humulus lupulus* preparations. J Res Pharm. 2026; 30(4): 1148-1156.

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ISSN: 2630-6344

https://doi.org/10.12991/jrespharm.1924663

Humulus lupulus L. (hop) is widely known in the brewing industry worldwide. Additionally, *H. lupulus* has established medicinal applications, particularly in treating sleep disorders as mild sedatives and stimulating gastric activity. The female inflorescences of *H. lupulus* contain high levels of polyphenolic compounds and acylphloroglucinols, where *in vivo* and clinical studies showed that *H. lupulus* flowers have a positive effect on anxiety [11-13]. Central nervous system disorders, particularly anxiety and depression, represent prevalent conditions, for which highly effective therapeutic strategies remain elusive.

In this present study, European Pharmacopeia grade *H. lupulus* flower extract, and *L. angustifolia* essential oil were evaluated for their potential *in vitro* monoamine oxidase MAO-A and MAO-B inhibitory effects. In addition to individual MAO inhibitions, the potential of extract and essential oil combinations was evaluated using the checkerboard method. To the best of our knowledge, this is the first study of the *in vitro* anxiolytic activity evaluation of standardized lavender and hops preparations and their combinations.

2. RESULTS AND DISCUSSION

2.1. Standardization

Phytochemical analysis of *L. angustifolia* essential oil was performed using GC/MS. The main components of commercially available essential oil were determined as 22.4% linalool and 19.2% linalyl acetate. Other components of lavender oil included camphor (17.9%), 1,8-cineol (12.3%), borneol (3.8%), *trans*-linalool oxide (3.0%), and *cis*-linalool oxide (2.5%). The phytochemical composition results of this present study were comparable and compatible with the European Pharmacopeia in terms of its linalol and linalyl acetate, whereas the camphor amount was relatively higher [14].

Table 1. GC/MS analysis results of the lavender oil

RRI	Compounds	relative%
1032	α-Pinene	0.3
1076	Camphene	0.7
1118	β-Pinene	0.2
1174	Myrcene	0.2
1203	Limonene	0.4
1213	1,8-Cineole	12.3
1246	(Z)-β-Ocimene	tr
1280	p-Cymene	0.4
1353	Hexyl isobutyrate	0.1
1386	Octenyl acetate	0.4
1424	Hexyl butyrate	0.2
1450	*trans*-Linalool oxide (Furanoid)	3.0
1478	*cis*-Linalool oxide (Furanoid)	2.5
1532	Camphor	17.9
1553	Linalool	22.4
1565	Linalyl acetate	19.2
1583	α-Santalene	0.7
1591	Bornyl acetate	0.2
1612	β-Caryophyllene	1.4
1617	Lavandulyl acetate	1.9
1616	Hotrienol	1.6
1686	Lavandulol	1.4
1690	Lavender lactone	0.2
1706	α-Terpineol	1.0
1719	Borneol	3.8
1733	Neryl acetate	0.2
1750	*cis*-Linalool oxide (Pyranoid)	0.3
1765	Geranyl acetate	0.4
1770	*trans*-Linalool oxide (Pyranoid)	0.3
1786	ar-Curcumene	0.3
1808	Nerol	tr
1857	Geraniol	0.2
1864	p-Cymen-8-ol	0.3
1961	3,7-Dimethyl-1,5-octadiene-3,7-diol	0.3
2008	Caryophyllene oxide	0.9
2104	Viridiflorol	0.1

RRI: Relative retention indices calculated against *n*-alkanes. % calculated from FID data

According to previous reports on *L. angustifolia* essential oil, the major components were linalol, linalyl acetate, and lavandulyl acetate as the major compounds [15-17],which is also in agreement with the present results.

In the present study, the dried flowers of *H. lupulus* were extracted with 70% ethanol. The reference compound humulone was used as the standard for the HPLC analyses of *H. lupulus* extract. According to Figures 1, and 2, humulone, was analysed with the retention time at 20.724, with 0.9% presence within the *Humulus lupulus* aquous ethanol extract.

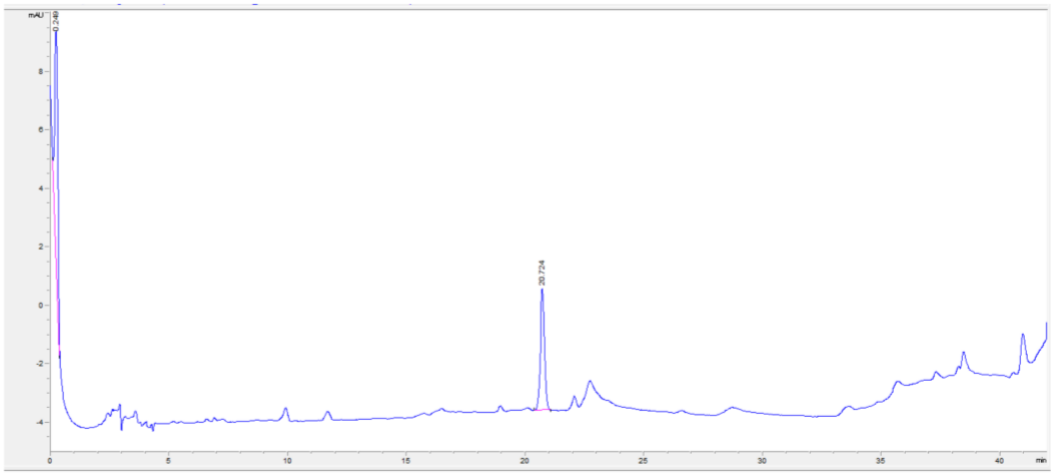

Figure 1. HPLC chromatogram of humulone (tR: 20.724)

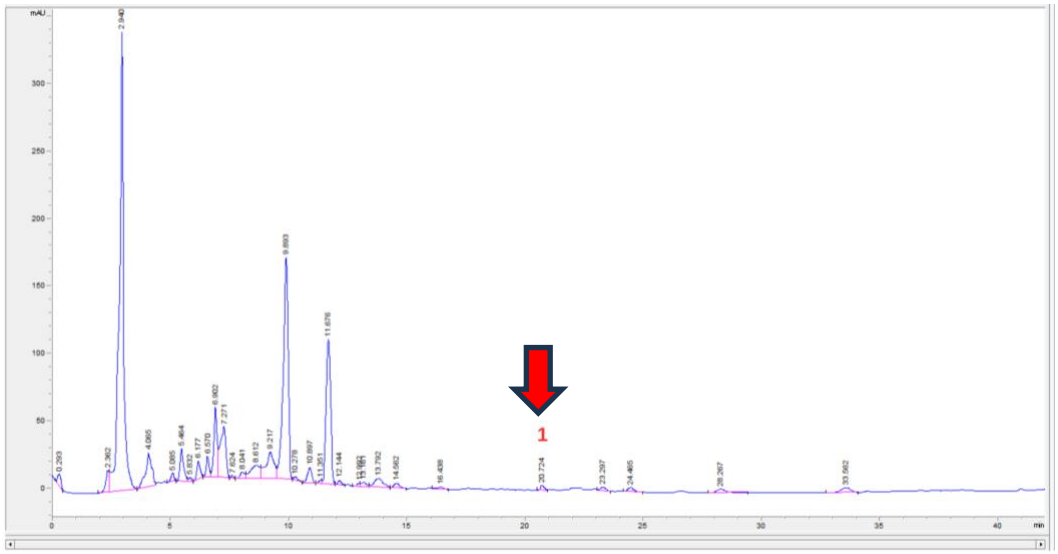

Figure 2. HPLC chromatogram of *H. lupulus* 70% ethanol with humolone (1)

H. lupulus is rich in phenolic secondary metabolites. The female inflorescences are used in the beer brewery. Due to its phytochemical diversity, it has a wide range of therapeutic effects [18]. Bitter acids, one of the main chemical groups of *H. lupulus*, are classified as α- and β- acids. The α- acids are humulone, cohumulone, and adhumulone, while β- acids are lupulone, colupulone, and adlupulone. There are minor bitter acids represented by post- and pre- homologs [19]. *H. lupulus* is also rich in kaempferol, quercetin, quercitrin, rutin, catechin gallate, and epicatechin gallate [20]. A previous study reported the characterization of bioactive compounds of hop in methanol and *n*-hexane extracts using the HPLC analyses. Xanthohumol was detected only in the methanol extract, where humulone, lupulone, and their homologs were observed in both extracts in varying percent values [21].

2.2. Enzyme inhibition of standardized plant materials

The anxiolytic effects of the ethanolic extract of *H. lupulus* and lavender oil were tested using *in vitro* MAO A and MAO B inhibitory assays. Based on the results of these analyses, the ethanol extract and essential oil resulted with IC_{50} values of 47.70 ± 1.45 µg/mL and 69.39 ± 2.07 µg/mL for MAO-A, respectively. Also, MAO-B inhibitions of the ethanol extract and essential oil showed IC_{50} values of 50.03 ± 1.02 µg/mL and 55.13 ± 1.62 µg/mL, respectively (Table 2).

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Table 2. IC_{50} values of the *H. lupulus* ethanol extract and *L. angustifolia* essential oil

	L. angustifolia (µg/mL)	*H. lupulus* (µg/mL)
MAO A	69.39 ± 2.07	47.70 ± 1.45
MAO B	55.13 ± 1.62	50.03 ± 1.02

Initial concentrations were selected based on the IC_{50} values of the aqueos extract and essential oil, and the MAO-A and MAO-B inhibitory effects of their combinations were evaluated using the checkerboard method. The highest MAO-A inhibition was observed in the combination containing *H. lupulus* at a 1:4 dilution and *L. angustifolia* at a 1:3 dilution, with an inhibition rate of 95.5%. In contrast, the highest MAO-B inhibition was obtained with the combination of *H. lupulus* at a 1:6 dilution and *L. angustifolia* at a 1:2 dilution, resulting in 57.5% inhibition. Detailed MAO-A and MAO-B inhibition of combinations were illustrated in Tables 3 and 4.

Table 3. Results of the checkerboard assay (MAO A)

		H. lupulus extract											
Combinations		1:1	1:2	1:3	1:4	1:5	1:6	1:7	1:8	1:9	1:10	1:11	1:12
L. angustifolia oil	1:1	64.30	72.96	79.47	84.16	88.35	80.72	73.14	45.30	40.92	33.16	22.80	20.64
	1:2	68.65	74.30	83.18	92.16	90.53	86.77	77.43	65.16	42.49	40.73	ND	13.18
	1:3	85.79	90.12	94.32	**95.50**	92.37	88.26	80.31	75.69	60.65	ND	33.16	19.82
	1:4	78.65	84.72	92.64	94.78	93.23	87.25	79.83	63.16	55.43	32.05	21.49	12.14
	1:5	71.23	82.05	86.99	88.55	90.13	81.27	73.30	ND	53.54	40.02	20.31	15.16
	1:6	64.66	77.43	79.96	80.16	72.66	64.95	60.34	52.96	43.40	31.29	19.14	11.93
	1:7	70.09	73.44	66.45	50.09	44.47	40.67	33.21	29.30	20.07	18.72	14.69	14.55
	1:8	55.13	40.08	33.94	37.43	30.42	27.69	20.05	17.14	12.16	10.02	13.93	12.47

ND: Not detected

Table 4. Results of the checkerboard assay (MAO B)

		H. lupulus extract											
Combinations		1:1	1:2	1:3	1:4	1:5	1:6	1:7	1:8	1:9	1:10	1:11	1:12
L. angustifolia oil	1:1	25.98	28.62	37.19	41.55	**54.07**	36.28	30.41	22.17	20.72	16.83	7.74	ND
	1:2	30.23	24.04	21.97	37.68	44.80	**57.50**	40.42	33.76	27.83	15.90	10.02	6.60
	1:3	41.56	40.07	45.60	51.44	**55.30**	35.17	31.60	25.08	20.90	ND	14.09	10.16
	1:4	42.30	47.66	50.13	48.88	43.75	39.14	33.17	26.05	17.78	14.90	10.30	7.17
	1:5	ND	43.39	46.14	40.76	37.23	34.87	30.38	24.90	18.16	11.09	6.92	3.16
	1:6	17.18	30.19	34.17	28.66	25.17	20.28	17.16	14.98	11.99	5.83	ND	ND
	1:7	19.76	27.05	ND	24.21	20.04	18.37	14.28	11.99	9.24	7.88	3.12	ND
	1:8	13.82	20.21	21.49	25.11	16.14	ND	10.32	6.77	4.83	2.18	ND	ND

https://doi.org/10.12991/jrespharm.1924663
J Res Pharm 2026; 30(4): 1148-1156

ND: Not detected

As it is well known, MAO is an enzyme that catalyzes oxidative deamination of biogenic amines such as dopamine, serotonin, norepinephrine, tyramine, and tryptamine, which are found in the outer mitochondrial membranes of the brain, liver, and intestinal mucosa. There are two isoenzymes, "MAO-A" and "MAO-B". "MAO-A"metabolizes amines such as serotonin, adrenaline, and noradrenaline, while "MAO-B"metabolizes dopamine, tryptamine, and tyramine [22]. The inhibition of "MAO-A"and "MAO-B"are utilized for the treatment of nervous system disorders such as depression, Parkinson's, panic disorder, and anxiety [23].

The central nervous system-related effects of *L. angustifolia* essential oil and its major compounds were investigated; however, no "MAO-A" inhibitory activity was reported [24]. Flavonoids present in *L. angustifolia* extract were demonstrated to inhibit "MAO-A", an enzyme responsible for the degradation of serotonin in the brain [25]. As the Committee on Herbal Medicinal Products mentioned in the monograph, *H. lupulus* is a herbal medicinal product for the relief of mental stress [26]. Owing to its sedative effects, hop was investigated in both *in vivo* and *in vitro* studies, and its "MAO-B"nhibitory activity suggested a promising potential in the treatment of mental disorders [27]. In another study conducted with ethanol extract of *H. lupulus*, the IC_{50} value was reported to range from 4 to 7 mg/mL [28]. Lupulone and humulone were reported as responsible substance on mental health improvement [29]. The synergistic effect plays an important role in determining the therapeutic effects of medicinal plant extracts. The synergistic effect increases bioavailability while protecting against toxicity [30]. Although *L. angustifolia* essential oil and *H. lupulus* extract were investigated individually for nervous system-related effects, to the best of our knowledge this is the first study on the combination for MAO inhibitory activity evaluation.

Overall, the experimental data obtained from phytochemical and *in vitro* biological activity evaluations showed that these herbal preparations have a significant synergistic effect in their combinations. To the best of our knowledge, in this present study, lavender and hops were evalutaed for the first time for their potential synergistic effects using binary combinations.

4. CONCLUSION

As a conclusion the combination of herbal preprations including the use of nano- and micro- systems may be more effective on the central nervous system with higher efficacy. However, future *in vivo* and clinical studies studies including toxicological assays are needed to confirm the safey.

5. MATERIALS AND METHODS

5.1. Materials and Extraction

Humulus lupulus L. dried flower and *Lavandula angustifolia* Mill. essential oil was obtained from commercial sources (St. Wolfgang Apotheke, Kümmersbruck, Germany). Other pure compounds, organic solvents, and chemicals were acquired from Sigma-Aldrich and Merck companies. The dried plant material was macerated using 70% ethanol at room temperature for 24 h. The extract was filtered and concentrated using an evaporator (Heidolph, Germany).

The fluorometric monoamine oxidase A/B inhibition screening kit was obtained from BioVision® (San Francisco, CA, USA). The kit provided pre-formulated assay components, including enzyme assay buffer, OxiRed Probe prepared in DMSO, lyophilized MAO A/B enzymes, lyophilized MAO A/B substrates, lyophilized developer, and lyophilized inhibitor controls consisting of selegiline and clorgiline.

5.2. Standardization

The *H. lupulus* ethanol extract was analyzed by HPLC versus humulon. HPLC analyses were performed using a Shimadzu 20A HPLC system. UV chromatograms were recorded using a Shimadzu SPD MOA DAD detector. The prepared extract was studied at a concentration of 10 mg/mL, and after dissolving it in ethanol, it was filtered through a 0.22 µm membrane filter for analysis. A C18 column (100 x 4.6 mm, 10 um) was used as the stationary phase, while the mobile phase consisted of A: Water: formic acid (100:0.1, v/v), B: Acetonitrile, and was studied as a linear gradient flow from 10% B to 78% B concentration between 0-18 minutes. In the flow, 18-21. minutes, the concentration was reduced from 90% B to 10% B (initial conditions). The flow rate was set to 1.2 mL/min. The injection volume was set to 5 µL, and the column temperature to 26°C [31].

The GC/MS analysis of *L. angustifolia* essential oil was carried out with an Agilent 5975 GC-MSD system. Innowax FSC column (60 m x 0.25 mm, 0.25 µm film thickness) was used with helium as carrier gas (0.8 ml/min). GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, and kept constant at 220°C for 10 min and then programmed to 240°C at a rate of 1°C/min. Split ratio was adjusted at 40:1. The injector temperature was set at 250°C. Mass spectra were recorded at 70 eV. The mass range was from *m/z* 35 to 450. The GC analysis was carried out using an Agilent 6890N GC system. FID detector temperature was 300°C. Relative percentage (%) amounts of the separated compounds were calculated from the FID chromatograms. Identification of the essential oil components were carried out by comparison of their relative retention times (RRT) with those of authentic samples or by comparison of their relative retention index (RRI) to series of *n*-alkanes. Computer matching against commercial (Wiley GC/MS Library, MassFinder Software 4.0) and the in-house "Başer Library of Essential Oil Constituents" built up by genuine compounds and components of known oils were performed [32].

5.3. Enzyme Assay

The studies were conducted using the method previously used by the team [33]. The plant extract and essential oil at various concentrations (ranging from 1 to 1000 µg/mL) were studied. The buffer, enzyme, and inhibitor were prepared according to the procedures of the commercial test kit. The extract and essential oil were incubated for 30 min at the same temperatures mentioned above. The fluorescence was kinetically measured using 535/587 nm (excitation/emission) at 5-min intervals.

5.4. Enzyme inhibition of combinations

The extract and essential oil were studied separately; the combined MAO-A and MAO-B enzyme inhibition of both the extract and the essential oil was investigated at different concentrations using the checkerboard technique. Lavender oil and hop extracts were prepared at individually determined IC_{50} values and serially diluted at ratios ranging from 1:1 to 1:12. The diluted samples were combined in a 96-well plate, and the enzyme assay was performed according to the kit protocol, and the ratios of inhibition were calculated. These combination activity studies were modified according to Karadağ et al.[17]

Acknowledgements: This study was supported by Anadolu University Scientific Research Projects Commission under the grant number BAP 2212S205.

Author contributions: Concept – F.D.; Design – F.D.; Supervision – F.D.; Materials – F.D.; Data Collection and/or Processing – F.D., S.E.K.; Analysis and/or Interpretation – F.D., S.E.K.; Writing – S.E.K., F.D.;

Conflict of interest statement: The authors have no conflicts of interest to declare.

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