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Research Article

The relaxant and contractile effects of *Verbascum speciosum* extracts on rat trachea: phenolic and organic acid contents and acetylcholinesterase inhibition

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ABSTRACT

Verbascum speciosum is traditionally used against respiratory system diseases such as asthma and bronchitis. This study investigated the effects of extracts prepared from *V. speciosum Schrad*. on isolated rat trachea. Plants collected in July 2023 were used to prepare flower methanol extract (FME), leaf methanol extract (LME), flower water extract (FWE), and leaf water extract (LWE). Phenolic and organic acid contents and acetylcholinesterase (AChE) inhibitory activity were measured. Methanol extracts had a more concentrated phenolic content. The IC_{50} values for AChE inhibition were higher in the methanol extracts. The extracts were directly tested on isolated rat trachea. LME did not produce contractile or relaxant responses. However, FME, FWE, and LWE elicited contractile responses in a dose- and extract-dependent manner. All extracts relaxed rat trachea pre-contracted with KCl and carbachol (CCh) in a dose- and extract-dependent manner. For trachea pre-contracted with ACh, LWE and FWE induced contraction, while LME and FME caused relaxation. LME exhibited the strongest relaxant effect at 100 mg, relaxing KCl-induced contraction by 103.81±7.94%, CCh-induced contraction by 109.21±2.64%, and ACh-induced contraction by 111.45±5.95%. The findings suggest that *V. speciosum* may contain various pharmacologically active compounds useful for treating respiratory diseases. However, the contractile response of water extracts to ACh-induced contraction may indicate potential risks of consuming *Verbascum*-containing herbal teas.

Keywords: Airway smooth muscle, Isolated organ bath, Rat trachea, *Verbascum, Verbascum speciosum* Schrad

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Introduction

Medical and aromatic plants have been used by humans for various diseases since ancient times. Today, these plants are utilized in various fields such as food, pharmaceuticals, cosmetics, and agriculture. The rate of usage of medicinal plants varies depending on the level of development of countries. A quarter of the pharmaceutical drugs used today are derived from plants (19).

Species of the genus *Verbascum*, belonging to the family Scrophulariaceae, have been traditionally used for treating various diseases since antiquity. These herbaceous plants, which are rarely annual but predominantly biennial or perennial, grow naturally across a wide geographical range, including Asia, Europe, and North America (4). The leaves and flowers of the plant are used for a broad spectrum of ailments, particularly respiratory disorders such as bronchial obstruction, irritating cough, dry cough, tuberculosis, asthma, pertussis, colds, and hoarseness. They are also employed for skin diseases, colic, hemorrhoids, and allergies and are preferred as expectorants and urinary relaxants (26). The species *Verbascum speciosum* Schrad., used in this study, is traditionally used against shortness of breath in the Yozgat and Sivas regions of Turkey, and against cough in the Hakkari region. The plant's leaves or flowers are used to prepare herbal tea for consumption (17, 24).

V. speciosum Schrad. contains numerous chemical compounds, including verbascoside, p-coumaric acid, fumaric acid, and apigenin. It has a high total phenolic and flavonoid content and possesses antioxidant activity comparable to standard antioxidants. It also exhibits high inhibitory activity against AChE, α-glucosidase, and α-amylase enzymes (22). Many studies have explored the chemical composition, antioxidant, and antimicrobial properties of *Verbascum* species (5, 15, 25, 28). Investigations into the antiviral and anticancer effects of *Verbascum* species have also been conducted (13, 20, 23). In isolated organ bath systems, the effects of *Verbascum latisepalum* Hub.-Mor. on rat aorta (9) and *Verbascum thapsus* on rabbit jejunum (3) have been studied. However, despite its widespread use for respiratory diseases, no study has examined the effects of *Verbascum* on isolated rat trachea. The aim of this study is to investigate the relaxant-contractile effects of water and methanol extracts prepared from the leaves and flowers of *V. speciosum* on isolated rat trachea. Furthermore, the study seeks to reveal the pharmacological potential of *V. speciosum* against respiratory diseases such as asthma.

Materials and Methods

Plant Collection and Identification: *V. speciosum* was collected in July 2023 from Kızılcahamam, Ankara Province (coordinates: 40°33'41.6"N, 32°39'57.4"E, 1048 m). The species and genus were identified at the Herbarium of the Ankara University Faculty of Science. A voucher specimen was deposited in the herbarium (voucher code: ANK-21104).

Plant Processing and Extraction: The flowers and leaves of the plants were separated and placed on drying papers with intervals to ensure proper airflow. Drying was carried out at room temperature in the shade. The leaves were flipped daily, and the flowers were stirred. After drying, the leaves and flowers were powdered using a mechanical grinder (Bot Waring, Commercial Blender).

Preparation of Methanol Extracts: Ultrasound-assisted extraction was performed using an ultrasonic bath (Elma D-78224) with a fixed frequency of 60 kHz. A total of 230 g of flowers and 230 g of leaves were subjected to static maceration in 2,300 mL of methanol at room temperature for 24 hours. The mixtures were then sonicated for one hour in the ultrasonic bath. The extracts were filtered using pleated filter paper to separate the filtrates and residues. The solvent was removed under reduced pressure at 30 °C using a rotary evaporator (Heidolph). As a result, 29 g of leaf methanol extract (LME) and 37 g of flower methanol extract (FME) were obtained. The extracts were stored in dark-colored glass containers at +4 °C until experimentation (21).

Preparation of Water Extracts: Ultrasound-assisted extraction was performed using an ultrasonic bath (Elma D-78224) with a fixed frequency of 60 kHz. A total of 230 g of flowers and 195 g of leaves were subjected to static maceration in 2,300 mL of water at room temperature for 24 hours. The mixtures were then sonicated for one hour in the ultrasonic bath. The extracts were filtered using pleated filter paper to separate the filtrates and residues. The filtrates were frozen at -80 °C and lyophilized using a freeze-dryer (Christ Gamma 2-16 LSC) at 0.04 mbar and -50 °C for 120 hours until completely dry. As a result, 18 g of leaf water extract (LWE) and 26 g of flower water extract (FWE) were obtained. The extracts were stored in dark-colored glass containers at +4 °C until experimentation (21).

Phenolic and Organic Acid Analyses: High-performance liquid chromatography (HPLC) was used to quantify phenolic compounds and organic acids in the crude extract. Phenolics were analyzed following Bozkurt et al. (10) using a 1260 Infinity II system (Agilent, USA) equipped with an ACE Generix 5 C18 column (4.6 × 250 mm). The mobile phase consisted of 0.1 % phosphoric acid in water (solvent A) and 100 % acetonitrile (solvent C). The flow rate was 0.8 mL/min, the column temperature was 30 °C, and the injection volume was 10 µL. Detection was performed at 300 nm with a reference wavelength of 500 nm. Organic acids were determined according to Aksoy et al. (1) using an Agilent Hi-Plex H ion exchange column (300 × 7.7 mm). The mobile phase was 0.02 N sulfuric acid in water, applied under isocratic conditions at a flow rate of 0.6 mL/min. The column temperature was maintained at 50 °C, with a 10 µL injection volume. Detection was conducted at 210 nm with a reference wavelength of 400 nm using a diode array detector. The calibration curves of phenolic compounds demonstrated high linearity, with correlation coefficients (R^2) ranging from 0.99887 to 1.00000. The limits of detection (LOD) and quantification (LOQ) were calculated using the formulas LOD = 3.3 σ/m and LOQ = 10 σ/m, where σ is the standard deviation of the response and m is the slope of the calibration curve. The LOD and LOQ values ranged from 0.12 to 9.72 ng/µL and 0.38 to 29.42 ng/µL, respectively.

Enzyme Inhibition Assays: The AChE inhibitory activity of the extracts was measured using the Ellman method. Acetylthiocholine iodide was used as the substrate, and 5,5-dithio-bis (2-nitrobenzoic acid) served as the reagent. Thiocholine released by enzymatic hydrolysis was measured spectrophotometrically at 412 nm. Tacrine, an AChE inhibitor, was used as the reference compound (18).

Animals: During the experiments, 80 male Wistar albino rats aged 16–18 weeks, weighing between 250–300 g were used. The rats were housed in groups of five in cages with a 12-hour light/dark cycle at the Hüseyin Aytemiz Experimental Research and Application Center of Kırıkkale University. They were provided pelleted feed and water *ad libitum*.

Tissue Preparation: The rats were anesthetized with an intraperitoneal injection of 10 mg/kg xylazine and 90 mg/kg ketamine. The tracheae were isolated, cleaned of surrounding tissues, and cut into 3–4 mm rings. The rings were placed in Petri dishes containing modified Krebs-Henseleit solution (118 mM sodium chloride [NaCl], 4.7 mM potassium chloride [KCl], 11 mM glucose [$C_6H_{12}O_6$], 25 mM sodium bicarbonate [$NaHCO_3$], 1.2 mM potassium phosphate [KH_2PO_4], 1.2 mM magnesium sulfate [$MgSO_4$], 1.3 mM calcium chloride [$CaCl_2$], and 0.05 mM ethylenediaminetetraacetic acid [EDTA], pH 7.4). Each tracheal ring was mounted in an isolated organ bath system (Commat, Turkey) using hooks in chambers containing 10 mL modified Krebs-Henseleit solution under 1 g of resting tension. Temperature was maintained at 37 °C with a heating circulator, and a gas mixture of 95% O_2 and 5% CO_2 was continuously supplied to the organ bath. The tracheal rings were equilibrated for 45 minutes, with the solution replaced three times at 15-minute intervals. After equilibration, tissue viability was confirmed using 60 mM KCl. Viable tissues were equilibrated over 45 minutes by replacing the modified Krebs-Henseleit solution three times at 15-minute intervals, and experimental protocols were applied. Tension changes were measured with a transducer (Biopac Systems MP 35, USA) and recorded on a computer.

Experimental Protocols: A stock solution of 1 g extract in 5 mL distilled water was prepared for FME, FWE, and LWE. Since LME was insoluble in distilled water, a solution was prepared by dissolving 1 g of extract in 5 mL of a 20% dimethyl sulfoxide (DMSO)/distilled water solution (1 mL DMSO and 4 mL distilled water). The final DMSO concentration in the organ bath was 1%. The effects of the 20% DMSO/distilled water mixture were directly tested on the isolated rat trachea and under pre-contracted conditions using KCl, carbachol (CCh), and acetylcholine (ACh) (n = 6). The EC_{85} values for CCh and ACh were calculated using GraphPad Prism Software 6 (CCh EC_{85}: 1 x 10^{-6} M, ACh EC_{85}: 1 x 10^{-6} M, n = 6). For the control, cumulative volumes of 5 µL, 10 µL, 35 µL, 100 µL, 100 µL, and 250 µL of 20% DMSO/distilled water (for CCh protocols) or distilled water (for other protocols) were applied. Each extract was tested on both uncontracted tracheal rings and tracheal rings pre-contracted with KCl, CCh, or ACh at cumulative doses of 1 mg, 3 mg, 10 mg, 30 mg, 50 mg, and 100 mg/10 mL (3). Each dose was applied for 5-minute periods and the final bath volume was maintained at 10 mL for all experiments. All relaxations and contractions were expressed as a percentage of CCh, ACh or KCl-induced maximal contractile response.

Statistical Analysis: Data were analyzed statistically using IBM SPSS Statistics version 20. Normality tests were performed to determine whether the groups exhibited parametric or non-parametric distributions. Since the data followed a parametric distribution, comparisons were made using Student's *t*-test, one-way analysis of variance, and post hoc Duncan tests. Statistical significance was set at $P < 0.05$, $P < 0.01$, $P < 0.001$.

Results

Phenolic and Organic Acid Contents of V. speciosum Shrad.: Methanol extracts had a higher concentration of phenolics than water extracts. The analyzed phenolics were also found in the highest amount in LME. The analyzed organic acids, however, varied among the extracts (Table 1).

Table 1. The quantity of phenolics and organic acids (mg/g extract) in FME, LME, FWE, LWE by HPLC.

Compounds	FME	LME	FWE	LWE
Chlorogenic acid	0.227	0.318	ND	ND
Catechin hydrate	0.827	1.079	ND	0.256
Caffeic acid	0.128	0.103	0.385	0.450
Vanillin	0.205	ND	0.300	0.142
***p*-Coumaric acid**	0.659	1.397	0.560	0.098
Rutin	2.332	23.302	0.110	0.100
Ferulic acid	1.585	5.792	0.080	0.261
Hydroxycinnamic acid	3.729	0.062	0.254	0.132
Naringin	0.873	6.890	0.215	ND
***o*-Coumaric acid**	0.324	0.035	0.148	0.212
Rosmarinic acid	0.115	0.317	0.074	ND
Salicylic acid	0.198	3.398	ND	ND
Resveratrol	0.230	0.487	0.111	0.342
Quercetin	0.600	1.514	0.180	0.049
Cinnamic acid	0.230	ND	ND	0.255
Naringenin	0.619	0.354	0.188	0.058
4-Hydroxybenzoic acid	ND	0.216	ND	0.260
Succinic acid	28.532	ND	99.884	111.448
Lactic acid	ND	ND	65.273	ND
Citric acid	52.198	8.149	ND	ND
Tartaric acid	31.251	ND	ND	ND
Formic acid	0.921	112.832	ND	0.878
Acetic acid	37.782	ND	ND	ND
Malic Acid	ND	29.914	ND	ND
Propionic acid	ND	ND	ND	0.600

ND: Not detected. Below the limit of detection (LOD).

Enzyme Inhibition Assays of V. speciosum Shrad.: The IC_{50} values for AChE inhibition were determined as follows: 0.001025 µg/mL for FME, 0.000949 µg/mL for LME, 0.001179 µg/mL for FWE, and 0.001098 µg/mL for LWE. These results were compared with tacrine, a known AChE inhibitor, which had an IC_{50} value of 0.000725 µg/mL. Methanol extracts exhibited lower IC_{50} values than water extracts (Table 2).

Table 2. IC_{50} values of FME, LME, FWE, LWE, and tacrine.

Extract	IC_{50} (µg/ml)	r^2
FME	0.001025	0.9860
LME	0.000949	0.9866
FWE	0.001179	0.9842
LWE	0.001098	0.9847
Tacrine (reference)	0.000725	0.9907

r^2: Good linearity.

Effect of Extracts on Uncontracted Rat Tracheal Rings: When applied alone, LME did not induce relaxation or contraction responses. However, LWE, FWE, and FME caused dose-dependent contraction responses at varying degrees. The contraction rate of FME showed little decreasing trend at dose 100 mg (Table 3).

Effect of Extracts on Rat Tracheal Rings Pre-Contracted with KCl: All extracts induced dose-dependent relaxation responses in tracheal rings pre-contracted with 60 mM KCl. The extent of relaxation was ranked as LME > FME > LWE > FWE at all doses. LME induced relaxation at all doses; FME and LWE induced relaxation at 30 mg, 50 mg, and 100 mg/10 mL doses; and FWE induced relaxation only at 50 mg and 100 mg doses (Table 4).

Effect of Extracts on Rat Tracheal Rings Pre-Contracted with CCh: In tracheal rings pre-contracted with 1×10^{-6} M CCh, all extracts caused dose-dependent relaxation. The order of relaxation was LME > FME > LWE > FWE across all doses. LME, FWE, and FME induced relaxation at all doses, while LWE showed relaxation at 10 mg, 30 mg, 50 mg, and 100 mg/10 mL doses (Table 5).

The 20% DMSO/distilled water mixture used as a control with the LME protocol induced relaxation at 10 mg, 30 mg, 50 mg, and 100 mg/10 mL doses. The synergistic effect of this mixture on the relaxation induced by LME was considered. Comparisons between relaxation responses of LME and the control group were statistically significant (Table 6).

Effect of Extracts on Rat Tracheal Rings Pre-Contracted with ACh: Methanol extracts (LME and FME) induced relaxation responses in tracheal rings pre-contracted with 1×10^{-5} M ACh (Table 7), while water extracts (LWE and FWE) caused contraction responses (Table 8, Figure 1, Figure 2). Separate statistical analyses were performed for methanol and water extracts.

Of all the extracts tested, LME at 100 mg demonstrated the highest relaxant activity on rat tracheal tissue. At a dose of 100 mg, LME relaxed KCl-induced pre-contraction by 103.81 ± 7.94%, CCh-induced pre-contraction by 109.21 ± 2.64%, and ACh-induced pre-contraction by 111.45 ± 5.95% (Figure 3).

Table 3. Contractile effects of LWE, FWE, and FME on uncontracted rat tracheal rings at various doses.

Dose	LWE % contraction	FWE % contraction	FME % contraction	P
10 mg	14.85 ± 4.56	_	10.95 ± 2.08	0.953
30 mg	16.46 ± 5.18	10.36 ± 1.85	24.54 ± 7.15	0.255
50 mg	15.69 ± 3.83	17.68 ± 3.64	29.05 ± 8.20	0.522
100 mg	20.94 ± 4.87	30.15 ± 7.42	26.69 ± 5.67	0.806

The one-way analysis of variance test was used to compare the 30 mg, 50 mg, and 100 mg/10 mL doses, and Student's t-test for the comparison of the 10 mg dose between LWE and FME. The difference in tension between LWE, FWE, FME was not statistically significant. Data are presented as mean ± standard error, n = 7 (number of trachea rings used).

Table 4. Relaxant effects of extracts on rat tracheal rings pre-contracted with KCl at various doses.

Dose	LWE % relaxation	LME % relaxation	FWE % relaxation	FME % relaxation	P
1 mg	_	19.74 ± 3.49	_	_	_
3 mg	_	29.95 ± 4.46	_	_	_
10 mg	_	36.35 ± 6.06	_	_	_
30 mg	6.84 ± 1.05^{b}	51.10 ± 5.69^{a}	_	18.03 ± 4.96^{b}	0.033
50 mg	14.80 ± 2.84^{c}	76.17 ± 7.24^{a}	8.88 ± 1.61^{c}	33.46 ± 6.00^{b}	<0.001
100 mg	41.26 ± 5.64^{c}	103.81 ± 7.94^{a}	24.25 ± 1.81^{c}	68.72 ± 8.13^{b}	<0.001

The one-way analysis of variance test followed by the post hoc Duncan test was used to compare the 30 mg, 50 mg, and 100 mg/10 mL doses. Different letters in the same row indicate statistically significant differences ($P < 0.05$). Data are presented as mean ± standard error, n = 7 (number of trachea rings used).

Table 5. Relaxant effects of the extracts on rat tracheal rings pre-contracted with CCh at various doses.

Dose	LWE % relaxation	LME % relaxation	FWE % relaxation	FME % relaxation	P
1 mg	_	19.42 ± 3.94[a]	3.56 ± 0.69[b]	11.37 ± 1.14[a]	0.01
3 mg	_	30.21 ± 2.97[a]	5.56 ± 0.93[b]	24.97 ± 3.88[a]	0.004
10 mg	14.13 ± 2.32[b]	56.45 ± 3.91[a]	8.19 ± 0.87[b]	42.07 ± 5.5[a]	<0.001
30 mg	30.17 ± 3.57[c]	89.16 ± 4.50[a]	13.66 ± 1.44[d]	62.12 ± 10.11[b]	<0.001
50 mg	36.51 ± 4.93[c]	104.35 ± 2.64[a]	27.65 ± 5.83[c]	81.09 ± 7.39[b]	<0.001
100 mg	72.33 ± 8.13[b]	109.21 ± 2.64[a]	58.48 ± 10.08[b]	101.23 ± 0.32[a]	<0.001

The one-way analysis of variance test followed by the post hoc Duncan test was used for all comparisons among the groups across doses. Different letters in the same row indicate statistically significant differences (P < 0.05). Data are presented as mean ± standard error. n = 7 (number of trachea rings used).

Table 6. Comparison of the relaxant effects of LME and control mixtures on rat tracheal rings pre-contracted with CCh at various doses.

Group	10 mg	30 mg	50 mg	100 mg
Control	9.63 ± 2.54	18.38 ± 4.87	28.65 ± 4.66	58.70 ± 4.45
LME	56.45 ± 3.91	89.16 ± 4.50	104.35 ± 2.64	109.21 ± 2.64
P	<0.001	<0.001	<0.001	0.001

The independent-samples t-test was used for comparisons. Data are presented as mean ± standard error. n = 7 (number of trachea rings used).

Table 7. Relaxant effects of methanol extracts on rat tracheal rings pre-contracted with ACh.

Dose	FME % relaxation	LME % relaxation	P
1 mg	12.91 ± 2.19	21.70 ± 4.25	0.110
3 mg	21.09 ± 2.66	30.47 ± 4.87	0.125
10 mg	23.43 ± 3.12	37.55 ± 5.90	0.064
30 mg	27.63 ± 3.22	55.39 ± 6.87	0.005
50 mg	37.72 ± 2.39	86.55 ± 6.39	<0.001
100 mg	67.70 ± 5.10	111.45 ± 5.95	<0.001

The independent-samples t-test was used for comparisons. Data are presented as mean ± standard error. n = 7 (number of trachea rings used).

Table 8. Contractile effect of water extracts on rat tracheal rings pre-contracted with ACh.

Dose	FWE % contraction	LWE % contraction	P
3 mg	6.50 ± 1.55	_	_
10 mg	20.66 ± 3.03	_	_
30 mg	43.79 ± 7.87	16.14 ± 3.59	0.100
50 mg	71.35 ± 11.48	30.66 ± 9.16	0.200
100 mg	85.14 ± 14.53	46.55 ± 10.68	0.058

The independent-samples t-test was used for comparisons. Data are presented as mean ± standard error. n = 7 (number of trachea rings used).

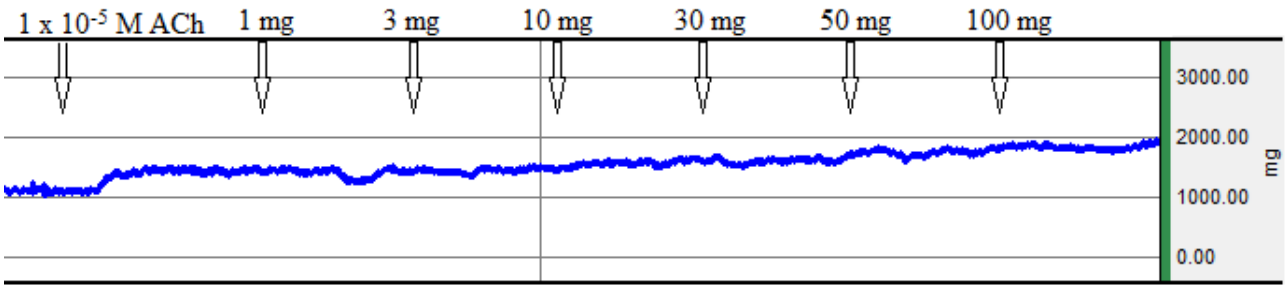

Figure 1. Original recording trace of LWE on rat tracheal rings pre-contracted with ACh at various doses.

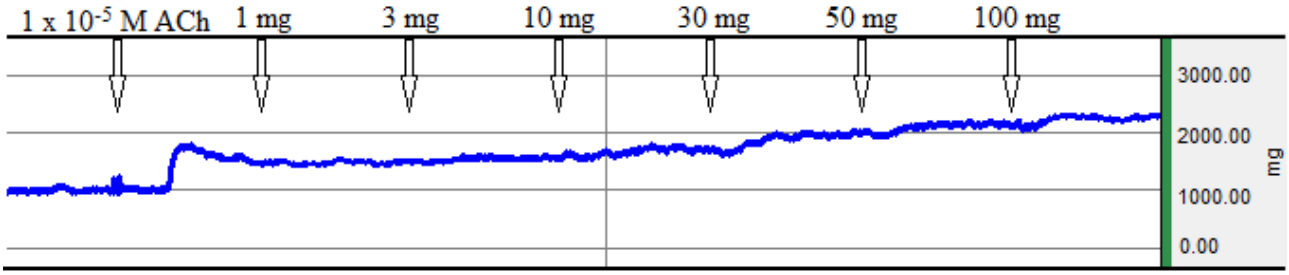

Figure 2. Original recording trace of FWE on rat tracheal rings pre-contracted with ACh at various doses.

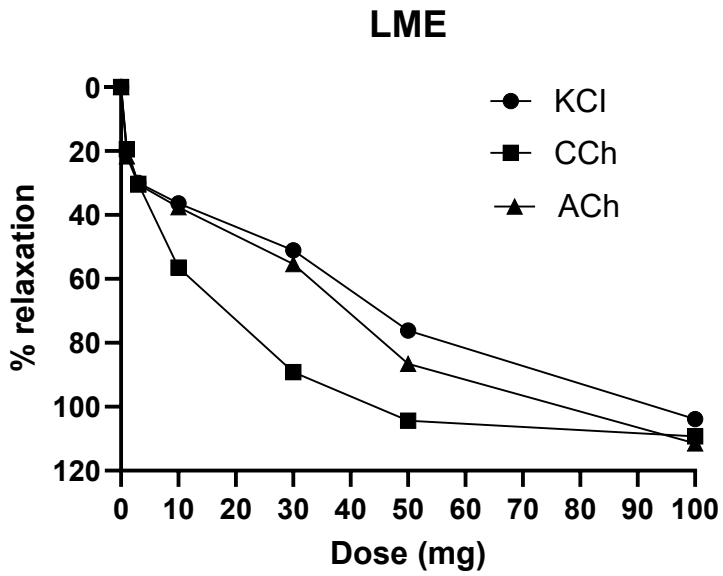

Figure 3. Relaxant effects of LME on rat tracheal rings pre-contracted with KCl, CCh, and ACh.

Discussion and Conclusion

Studies indicate that *Verbascum* species contain phenolic and flavonoid compounds in varying proportions. Consequently, antioxidant activity varies between species (6). These variations are also observed among different plant parts. In a study by Grigorov et al. (16) ethanol extracts were separately prepared from the flowers and leaves of *V. niveum*. The results revealed that different plant parts contained varying amounts of phenolic compounds. Higher levels of flavonoids were measured in leaf extracts compared to flower extracts. While some phenolic compounds were present in both extracts, others were identified only in the leaves or flowers. Supporting these results, in the current study, measurements of phenolic compounds in extracts prepared from the leaves and flowers of *V. speciosum* showed differences depending on the plant part.

The choice of solvent for extraction significantly affects the transfer of chemical compounds from the plant parts to the extract. In a study by Boğa et al. (8), petroleum ether, acetone, methanol, and water extracts of *V. flavidum* were analyzed for total phenolic and flavonoid content. The results demonstrated variations in phenolic and flavonoid levels among the extracts. The methanol extract contained the highest number of phenolic compounds, whereas the acetone extract exhibited the highest flavonoid content. The authors showed that extracts prepared from the same species with different solvents could have varying amounts of total phenolic and flavonoid compounds. Consistently, measurements performed in our study indicated that methanol extracts contained more phenolic compounds compared to water extracts.

Kızıltaş et al. (22) identified several phenolic compounds in the ethanol and water extracts of V. *speciosum.* In our measurements, unlike those of Kızıltaş et al. (22), catechin hydrate, resveratrol, hydroxycinnamic acid, vanillin, and ferulic acid were detected in the extracts. This discrepancy may be attributed to the use of aerial parts of the plant for extraction and the preparation of an ethanol extract by Kızıltaş et al. (22). Additionally, factors such as the soil composition where the plant was

collected, the collection time, climatic conditions, drying method, and extraction method are also believed to have contributed to this difference (6).

Regarding the presence of AChE inhibitors in *Verbascum* plants, Georgiev et al. (14) found that forsythoside B, leucoseptoside B, and verbascoside obtained from the methanol extract of *V. xanthophoeniceum* exhibited anticholinesterase activity. In another study, Alan and Almaz (2) prepared both ethanol and aqueous extracts from the leaves and roots of *V. insulare* and examined their anticholinesterase activities. All extracts demonstrated anticholinesterase activity. Angeloni et al. (7) prepared aqueous and methanol extracts from the aerial parts of *V. bombyciferum*. Measurements of anticholinesterase activity showed the highest activity in methanol extracts. In our study, the IC_{50} values against AChE were higher in methanol extracts, yielding results consistent with those reported by Angeloni et al. (7). Kızıltaş et al. (22) measured high anti-AChE activity (IC_{50}: 1.516 µg/mL) in ethanol extracts prepared from the aerial parts of *V. speciosum*. This result aligns with the findings of our study.

When applied alone to rat trachea, all extracts except LME induced a contraction response. This contraction is considered to result from the presence of AChE inhibitors in the extracts. In a study by Yıldırım (27), the application of the AChE inhibitor trichlorfon to isolated rabbit trachea caused contraction of the tissues. Similarly, our study suggests that the contractions observed in rat trachea tissue may have been induced by AChE inhibitors present in the extracts. Despite LME having the highest AChE inhibitory activity, the lack of contractile response may be attributed to the presence of dominant relaxant constituents that override the effects of AChE inhibitors.

In this study, all extracts induced dose-dependent relaxation responses in tracheal rings pre-contracted with 60 mM KCl. Ali et al. (3) found that the methanol extract of *V. thapsus* relaxed KCl-induced contractions in isolated organ baths with rabbit jejunum. In our study, KCl-induced tracheal contractions also exhibited relaxation responses. The contraction response of LWE and FWE to ACh-induced pre-contraction indicates that relaxing agents in the water extracts were either insufficient to suppress the AChE inhibitors or entirely absent. Despite the higher content of AChE inhibitors in methanol extracts compared to water extracts, the strong relaxation response obtained from methanol extracts suggests that the relaxant effect of phenolic compounds in methanol extracts overrides the effect of AChE inhibitors.

In rat tracheal rings pre-contracted with KCl, CCh, and ACh, LME resulted in the highest percentage of relaxation, followed by FME. The superior relaxant effect of methanol extracts can be attributed to methanol effectiveness as a solvent, which facilitates the extraction of more chemical compounds. The phenolic content analysis of the extracts in this study supports this conclusion. The highest phenolic content was observed in LME, followed by FME. In isolated organ bath experiments, LME produced the greatest relaxation effect, followed by FME. These findings suggest that the relaxant effect on tracheal smooth muscle stems from the phenolic compounds present in the plant. The relaxant effects of certain phenolic compounds on rat trachea have been demonstrated in previous studies (12).

The relaxant effect of LME at a dose of 100 mg on pre-contractions induced by KCl, CCh, and ACh is noteworthy. In a study conducted in an isolated organ bath system, the β2 adrenergic receptor agonist bambuterol was tested on rat trachea pre-contracted with methacholine, and the highest relaxation response obtained from bambuterol at various doses was 115.9 ± 12.2% (11). Considering the percentage relaxation values of bambuterol, the precursor of terbutaline used in asthma treatment, it is considered that LME exhibits therapeutic potential against asthma.

Verbascum flowers and leaves are commonly used against respiratory diseases. They are consumed as tea or in extract form. While the experimental results support the traditional use of *Verbascum* against respiratory diseases, the contraction response of water extracts to ACh-induced pre-contraction indicates the need for caution in consuming teas made from the plant's leaves and flowers. Unintended effects may occur depending on the source and dosage of the contraction in individuals with respiratory disorders.

In conclusion, the experimental protocols demonstrated that the methanol extracts of *V. speciosum* had significant relaxant effects on rat trachea. The relaxation responses observed were directly proportional to the phenolic content of the extracts. This finding suggests that the phenolic compounds present in the plant may be the primary contributors to these relaxant effects. Despite methanol extracts containing higher levels of AChE inhibitors compared to water extracts, they exhibited the greatest relaxant effect. This indicates that the compounds responsible for the relaxant effects may override the effect of AChE inhibitors. Furthermore, it was observed that these compounds were more efficiently extracted into methanol than water. The contraction response of water extracts in trachea pre-contracted with ACh suggests that consuming herbal teas prepared from *Verbascum* leaves and flowers may pose risks for individuals with respiratory conditions.

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Ethical Statement

The experimental protocol was approved by the Kırıkkale University Animal Experiments Local Ethics Committee (decision number: 05, meeting date: February 23, 2023, meeting number: 2023/02).

Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

All authors designed the project. AT carried out the experiments. All authors contributed to the interpretation of the results. AT took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting the results of the manuscript are available by the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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