

Inhibitory Effect of the Methanolic Extract of *Verbascum latisepalum* Hub.-Mor. on Endothelium-Dependent Relaxation in Rat Thoracic Aorta

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The leaves and flowers of *Verbascum* species are used to treat respiratory disorders, haemorrhoids, rheumatic pain, and wounds as well as for the treatment of eczema and other types of inflammatory skin conditions in traditional Turkish medicine. We examined the effect of the methanolic extract of the aerial parts of *Verbascum latisepalum* Hub.-Mor. on the endothelium-dependent relaxation response in rat aortic rings which is mediated by nitric oxide (NO). Six fractions, A–F, were obtained from the methanolic extract through bioassay-guided fractionation procedures. The phenylethanoid glycoside verbascoside was isolated from fraction D and its structure elucidated by spectral techniques. The inhibitory effects of the extract, its fractions, and verbascoside on the acetylcholine-induced relaxation response in phenylephrine-precontracted aorta was examined in the absence and presence of L-arginine, a precursor in the synthesis of NO. The observation that the effects of the methanolic extract, of fraction D, and of verbascoside were reversed by L-arginine, indicates that verbascoside has an inhibitory effect on the synthesis of NO. This effect should be taken into consideration in view of the wide range of uses of *Verbascum* species in Turkish folk medicine.

Key words: *Verbascum latisepalum*, Nitric Oxide Synthase, Vasorelaxation

Introduction

A number of species of the Scrophulariaceae are valued for their curative properties and are widely used both in domestic and regular medicine. These plants are known as “sığır kuyruğu” in Anatolia (Baytop, 1999). The genus *Verbascum* is represented by 233 species, 196 of which are endemic in the Turkish flora (Huber-Morath, 1978; Ekim, 2000). The leaves and flowers of *Verbascum* species are reported to have expectorant, mucolytic, and demulcent properties, and are used to treat respiratory disorders such as bronchitis, dry coughs, tuberculosis, and asthma in traditional Turkish medicine. The species are also used to treat haemorrhoids, rheumatic pain, superficial fungal infections, wounds, and several types of inflamma-

tory skin diseases (Baytop, 1999; Turker and Camper, 2002; Turker and Gurel, 2005).

In our previous studies, antinociceptive and anti-inflammatory activities of eight endemic *Verbascum* species were investigated. The methanolic extracts of these species displayed significant antinociceptive activity in the *p*-benzoquinone-induced writhing test, and anti-inflammatory activity at 200 mg/kg body weight per os using carrageenan-induced and PGE₁-induced hind paw edema models and 12-*O*-tetradecanoyl-13-acetate (TPA)-induced mouse ear edema models (Tatli *et al.*, 2008; Akdemir *et al.*, 2011). Besides these, the methanolic extracts of thirteen *Verbascum* species growing in Turkey, including *V. latisepalum* Hub.-Mor., were also assessed for their *in vivo* wound-healing activity evaluated by linear incision and cir-

cular excision experimental models and subsequent histopathological analysis. The methanolic extract of *V. latisepalum* was found generally highly effective (Süntar *et al.*, 2010).

Phytochemical studies of these species have revealed the presence of phenylethanoid glycosides. Especially verbascoside is widely found in the genus *Verbascum* (Tatli and Akdemir, 2004). Phenylethanoid glycosides possess a number of biological activities such as antioxidant, anti-inflammatory, cytotoxic, anti-tumour, antiulcer, analgesic, antihypertensive, and immunosuppressive (Jimenez and Riguera, 1994). In addition, the nitric oxide (NO)-scavenging activity of phenylethanoids (verbascoside), which possibly contributes to their anti-inflammatory effects, has been demonstrated in lipopolysaccharide-stimulated murine macrophage cells (Xiong *et al.*, 2000).

NO is a highly reactive free radical which is involved in the regulation of many physiological and pathophysiological processes (Hallinan *et al.*, 2002; Ji *et al.*, 2009). It is synthesized from L-arginine by three different isoforms of the enzyme nitric oxide synthase (NOS). The endothelial (eNOS) and neuronal (nNOS) isozymes are constitutive, while the third isoform is inducible NOS (iNOS) and is expressed in response to pro-inflammatory stimuli such as cytokines or pathogens (Garcin *et al.*, 2008).

One of the important effects of NO is its vasorelaxant activity which is very critical in the regulation of vascular tone. In the vascular system it is synthesized in the endothelium and diffuses to the vascular smooth muscle inducing a relaxation response via a guanylate cyclase and cGMP-dependent pathway (Mayer and Hemmens, 1997). Therefore, blocking its effect may cause an increase in the vascular tone which leads to an elevation in blood pressure. This effect can be more significant in hypertensive condition. Considering the common systemic use of *Verbascum* species in traditional Turkish medicine, their vascular effects need to be determined. Here we investigated the effect of the methanolic extract of the aerial parts of *V. latisepalum* on the endothelium-dependent relaxation response in isolated rat thoracic aortae, and isolated and chemically characterized its active constituents through bioassay-guided fractionation procedures.

Material and Methods

Plant material

V. latisepalum Hub.-Mor. (Scrophulariaceae) was collected in a *Pinus brutia* forest between Burdur and

Cavdir in the Mediterranean region of Turkey during August 2002. Voucher specimens were authenticated by Prof. Hayri Duman, Gazi University, Faculty of Science, Etiler, Ankara, Turkey, and were deposited at the herbarium of the Faculty of Pharmacy, Hacettepe University, Ankara, Turkey (HUEF 02007).

Bioassay-guided fractionation and isolation of active ingredients

Preparation and fractionation of the MeOH extract

The air-dried and powdered aerial parts of *V. latisepalum* (327.3 g) were extracted twice with MeOH (2 × 2000 ml) at 40 °C for 12 h. After evaporation of the solvent of the combined extracts *in vacuo*, 34.9 g MeOH extract were obtained. The methanolic extract was fractionated by vacuum liquid chromatography (VLC) over a polyamide column (50–160 µm, 250 g; Fluka, Buchs, Switzerland), eluting with H₂O, followed by increasing concentrations of MeOH (0, 25, 50, 75, and 100%, 1600 ml) to afford six main fractions: fraction A, 4.50 g; fraction B, 1.34 g; fraction C, 2.52 g; fraction D, 2.63 g; fraction E, 383.0 mg; fraction F, 205.96 mg.

Chromatographic separation and isolation of the active constituents

Fraction D, which showed the highest activity for the inhibition of acetylcholine-induced relaxation response, was fractionated by VLC over a LiChroprep C₁₈ column (C-18, Sepalyte 40 µm, 150 g; Merck, Opfikon-Glattbrugg, Switzerland). A H₂O and MeOH mixture (30–100% MeOH) afforded seven subfractions: subfraction D₁, 46.5 mg; subfraction D₂, 147.7 mg; subfraction D₃, 55 mg; subfraction D₄, 43.1 mg; subfraction D₅, 66.2 mg; subfraction D₆, 40.8 mg; subfraction D₇, 13.3 mg. The solvent polarity was changed by increasing the MeOH content. Purification of subfraction D₂ (147.7 mg) by silica gel (230–400 mesh, 140 g; Merck) column chromatography [CHCl₃/MeOH (70:30→60:40)] furnished verbascoside (85 mg), which was identified by 1D and 2D NMR and HR-ESI mass spectrometry as described previously (Akdemir *et al.*, 2004).

Biological activity tests

Test animals

The study had been approved by Hacettepe University Animal Ethics Committee (No. 2008/29-7). Male

Sprague-Dawley albino rats, weighing 200–250 g, were used in the study.

Drugs and solutions

Phenylephrine hydrochloride, acetylcholine hydrochloride, *N*^ω-nitro-L-arginine methyl ester (L-NAME) hydrochloride, and papaverine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). All drug solutions were prepared in distilled water. The methanolic extract of *V. latisepalum* was dissolved in dimethylsulfoxide (DMSO). DMSO at concentrations matching those in test solutions did not alter the acetylcholine-induced relaxation responses.

Tissue preparation

Rats were killed by exsanguination, and their thoracic aorta was isolated. Ring preparations of approximately 2–3 mm in length were prepared. Isometric changes in tension were recorded with an isometric force transducer (MAY95-transducer data acquisition system; COMMAT, Ankara, Turkey). The composition of the Krebs-Henseleit solution was (in mM): NaCl (113), KCl (4.7), MgSO₄ (1.2), CaCl₂ (2.5), KH₂PO₄ (1.2), NaHCO₃ (25.0), glucose (11.6); it was gassed with 95% O₂/5% CO₂ at 37 °C and pH 7.4.

Experimental protocol

The aortic rings were mounted under a resting tension of 1.5 g in 10-ml organ baths filled with Krebs-Henseleit solution. Tissues were equilibrated for 1.5 h and washed with Krebs-Henseleit solution every 15 min before each experimental procedure.

The aortic rings were precontracted by 1 μM phenylephrine (60–80% of the maximum contraction), and then the relaxation response to acetylcholine (10 nM–0.1 mM) was evaluated by cumulative addition to the organ bath. The effects of the NOS inhibitor L-NAME (10⁻⁵ M), the methanolic extract of *V. latisepalum*, its fractions A–F, and verbascoside, which had been isolated from fraction D, were examined on acetylcholine-induced relaxation responses. For this purpose, the tissues were incubated for 30 min with one of the substances mentioned above prior to precontraction with phenylephrine for the acetylcholine relaxation response. In another group of experiments, L-arginine (1 mM) was added to the organ bath at the same time with either L-NAME, *V. latisepalum* extract, each of its fractions,

or verbascoside to evaluate if it would affect the inhibition of the relaxation responses. Each concentration-response curve to acetylcholine was obtained with individual preparations.

The relaxation response to acetylcholine was expressed as the percentage of papaverine (10⁻⁴ M)-induced relaxation. Papaverine was added to the organ bath after the maximum relaxation response to acetylcholine had been obtained.

Statistical analysis

Data are represented as means ± standard error of the mean (S.E.M.). Statistical analysis was done by ANOVA/Newman-Keuls and Student's *t*-test using GraphPad Prism4 software. *P* < 0.05 was considered as significant.

Results and Discussion

The effects of the methanolic extract of *V. latisepalum* on the acetylcholine-induced endothelium-dependent relaxation response in the rat thoracic aorta was examined. Rat thoracic aorta is a commonly used pharmacological preparation for determining the effect of test compounds on vascular relaxation (Pereira *et al.*, 2013; Qian *et al.*, 2012). Acetylcholine induces an endothelium-dependent relaxation response which is mediated by NO. NO plays an important role in the regulation of the vascular smooth muscle tone. It is mainly synthesized by eNOS in the endothelium and, after its release from endothelial cells, NO diffuses to the vascular smooth muscle cells and initiates vasodilation (Mayer and Hemmens, 1997). In the present study, a concentration-dependent relaxation response to acetylcholine (0.1 nM–0.1 mM) was obtained in isolated rat thoracic aorta (Fig. 1A). It is well established that this response is mainly mediated by NO in rat aorta (Palmer *et al.*, 1987; Moncada and Higgs 2006; Loscalzo, 2013). Furthermore, in our study the acetylcholine-induced relaxation response was inhibited by the NOS inhibitor L-NAME (10 μM), which is a pro-drug and releases the actual NOS inhibitor upon hydrolysis of the methyl ester (Fig. 1C). Increasing concentrations (3–30 μg/ml) of the methanolic extract of *V. latisepalum* inhibited the acetylcholine-induced relaxation response at 10 and 30 μg/ml (Fig. 1A). This inhibitory effect of the methanolic extract may occur through scavenging of NO, attenuation of the synthesis of NO through inhibition of eNOS, through damage inflicted to the

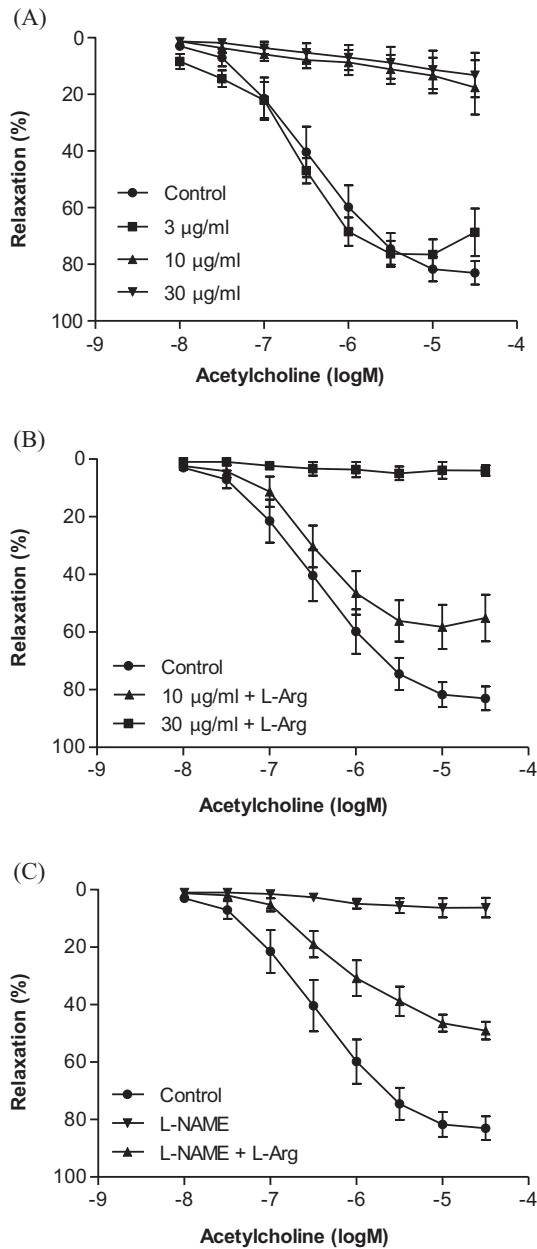


Fig. 1. (A) Concentration-dependent relaxation response to acetylcholine in the absence and presence of 3, 10, and 30 µg/ml *V. latisepalum* methanolic extract in isolated rat thoracic aorta. (B) Effect of 1 mM L-arginine (L-Arg) on the acetylcholine-induced relaxation response in the presence of 10 and 30 µg/ml *V. latisepalum* methanolic extract. (C) Effects of L-NAME (10 µM) and L-NAME (10 µM) + L-Arg (1 mM) on the acetylcholine-induced relaxation response in isolated rat thoracic aorta.

endothelium, or interference with the signal transduction of NO. To identify the likely mechanism of this inhibition, we examined the effect of L-arginine which is the precursor of NO. The competitive nature of L-arginine's action against NOS inhibitors has been shown previously (Hecker *et al.*, 1990; Cavalcante *et al.*, 2010; Förstermann and Sessa, 2012). L-Arginine (1 mM) reversed the inhibitory effect of the *V. latisepalum* extract at 10 µg/ml but not at 30 µg/ml (Fig. 1B). This finding suggests that the inhibitory effect of *V. latisepalum* methanolic extract at 10 µg/ml concentration is mainly due to the inhibition of eNOS which is the main enzyme responsible for the synthesis of NO in the endothelium. To confirm this effect, a similar experiment was performed with the NOS inhibitor L-NAME. The inhibition of the response to acetylcholine by L-NAME (10 µM) was also prevented in the presence of L-arginine in isolated rat thoracic aorta (Fig. 1C). This finding supports our conclusion that the methanolic extract causes *in vivo* eNOS inhibition at 10 µg/ml concentration. On the other hand, the inhibitory effect at higher concentration of the extract (30 µg/ml) may be due to damage to the endothelium, since it was not reversed by L-arginine. This damage may be caused by saponins which are probably present in this extract. Saponins have been isolated from several *Verbascum* species growing in Turkey (Tatli and Akdemir, 2004), and are known to impair endothelial cell functions and abolish the endothelium-dependent relaxation response through endothelium denudation (Samata *et al.*, 1986).

In the second part of the study, the effects of different fractions of the *V. latisepalum* methanolic extract were examined to identify the fraction responsible for the inhibitory effect on the acetylcholine-induced relaxation response. Six different fractions (by increasing polarity of the solvent) of the extract were obtained, and their effect on the relaxation response to acetylcholine was assessed. Inhibition of the response was obtained with fraction A at 10 µg/ml, fraction B at 30 and 100 µg/ml, fraction C at 10 µg/ml, fraction D at 10 and 30 µg/ml, fraction E at 30 and 100 µg/ml, and fraction F at 30 and 100 µg/ml (Figs. 2A–F).

Next, the effects of L-arginine on the inhibition brought about by these fractions were tested as described above. L-Arginine reversed the inhibitory effect of fraction D at 10 µg/ml but none of the effects of any other fraction, or of 30 µg/ml of fraction D (Figs. 2A–F). This finding indicates that only the action of 10 µg/ml of fraction D appears to be truly me-

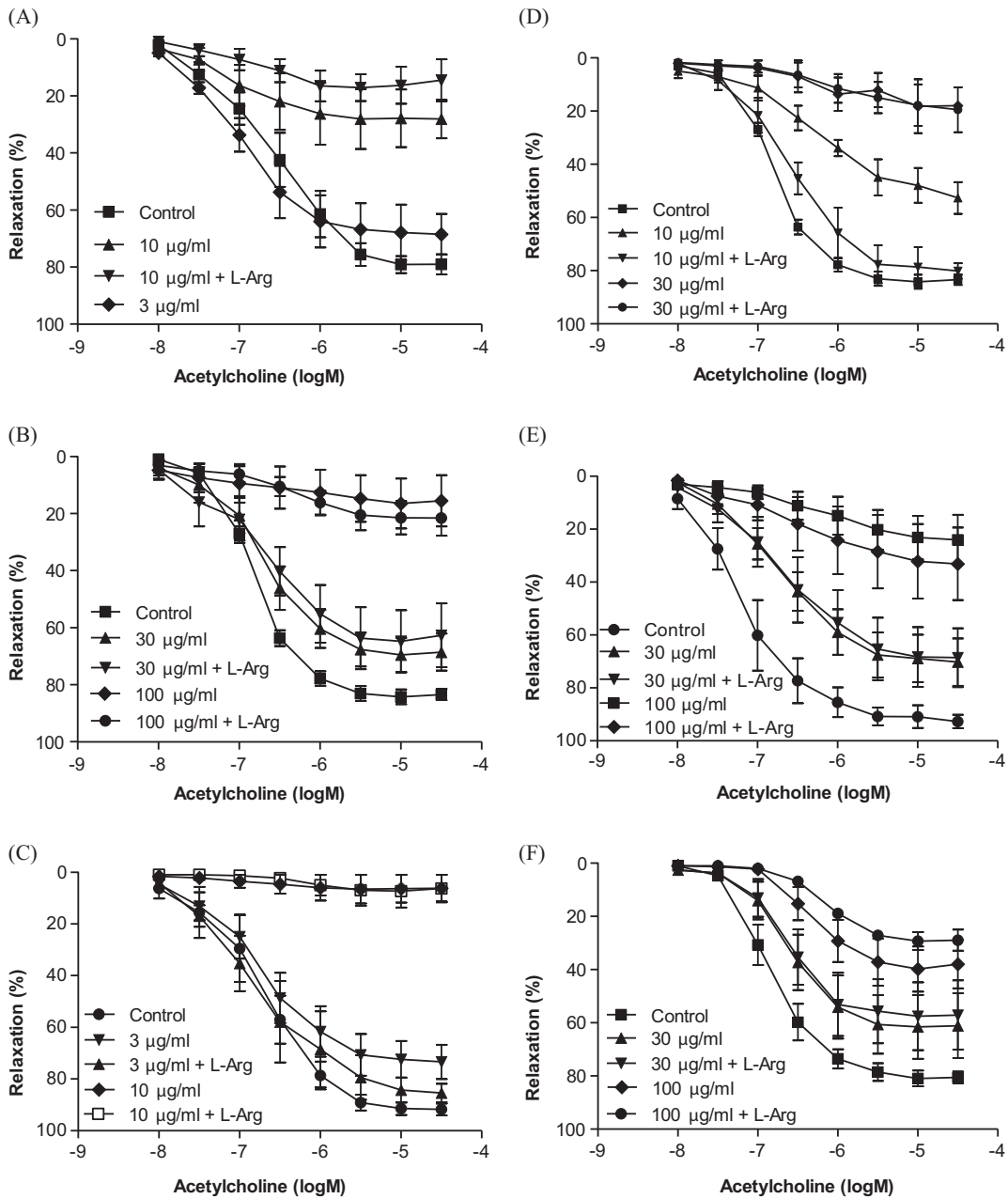


Fig. 2. Concentration-dependent relaxation response to acetylcholine in the absence and presence of different concentrations of (A) fraction A, (B) fraction B, (C) fraction C, (D) fraction D, (E) fraction E, (F) fraction F, and the effect of L-arginine (L-Arg) (1 mM) on these responses.

diated through inhibition of eNOS. The inhibitory effect of other fractions tested seems to be more likely mediated through saponin-induced endothelial damage rather than eNOS inhibition as suggested above for the

high concentration (30 $\mu\text{g/ml}$) of the methanolic extract itself.

Fraction D contains the biologically active compound verbascoside. This compound inhibited the re-

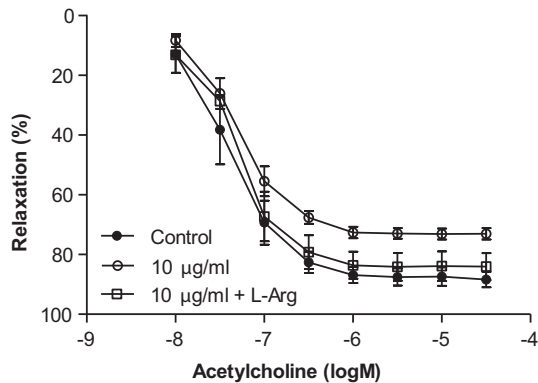


Fig. 3. Concentration-dependent relaxation response to acetylcholine in the absence and presence of 10 µg/ml verbascoside and 10 µg/ml verbascoside + 1 mM L-arginine (L-Arg) in isolated rat thoracic aorta.

laxation response at 10 µg/ml, and this inhibitory effect was attenuated in the presence of L-arginine (Fig. 3). This data again indicates that the inhibitory effect of verbascoside is due to the inhibition of eNOS, as suggested for the inhibitory effects of *V. latisepalum* methanolic extract and fraction D, both at 10 µg/ml.

An activity of verbascoside leading to *in vivo* NOS inhibition parallels findings of other studies. It has been demonstrated that verbascoside enhances the vasoconstriction response through inhibition of en-

dothelial NO formation (Tam *et al.*, 2002). In another study, verbascoside was shown to impair aortic relaxation partially through inhibition of endothelial NO production (Lau *et al.*, 2004). Besides inhibiting eNOS, verbascoside has also inhibitory activity on iNOS, which may be involved in the anti-inflammatory effect of *V. latisepalum* (Garcin *et al.*, 2008).

In conclusion, the inhibitory effects of the methanolic extract of *V. latisepalum*, its fraction D, and the biologically active compound verbascoside on acetylcholine-induced endothelium-dependent relaxation response seem to be mediated via the inhibition of eNOS. The finding that the NO precursor L-arginine attenuates this inhibition supports this hypothesis, because L-arginine also attenuates the effect of the NOS inhibitor L-NAME. However, there are possibly other substances in the extract that also counteract acetylcholine-induced responses. Because the methanolic extract, fraction D, and verbascoside all exerted comparable inhibitory activities at 10 µg/ml concentration, it appears that verbascoside has a low specific activity, and that there are additional compounds in the extract and fraction D that contribute to the inhibitory effect of acetylcholine-induced relaxation responses. Since there is a wide range for the traditional use of *Verbascum* species, the eNOS inhibitory effect of this plant should always be taken into consideration for patients with hypertension and other vascular diseases.

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