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Original Article

Endothelium-independent vasorelaxant effect of a *Berberis orthobotrys* root extract via inhibition of phosphodiesterases in the porcine coronary artery

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ABSTRACT

Background: *Berberis orthobotrys* Bien ex Aitch. (Berberidaceae) is a plant indigenous of Pakistan that is locally used for the treatment of hypertension.
Hypothesis: This study evaluated the vasoactive properties of a *Berberis orthobotrys* root extract and its fractions, and investigated the role of the endothelium and the underlying mechanism.
Study design: An aqueous methanolic extract of *Berberis orthobotrys* roots was prepared and submitted to a multi-step liquid-liquid fractionation with solvents of increasing polarity. Vascular reactivity of the different fractions was assessed using porcine coronary artery rings either with or without endothelium, and in the presence or absence of specific pharmacological tools. The ability of *Berberis orthobotrys* extracts to affect phosphodiesterase (PDE) activity was evaluated using a radioenzymatic method and purified phosphodiesterases.
Results: The aqueous methanol extract induced similar relaxations in coronary artery rings with and without endothelium, and, amongst the three derived preparations, the butanol fraction (BFBO) was slightly but significantly more effective than the ethyl acetate fraction and the aqueous residue in rings without endothelium. Analysis of the butanol fraction (BFBO) by LC-ELSD-MS indicated the presence of four major isoquinoline alkaloids including berberine. BFBO significantly potentiated the relaxations induced by cyclic GMP- and cyclic AMP-dependent relaxing agonists, and inhibited contractions to KCl, $CaCl_2$, and U46619 in endothelium denuded rings. In contrast, BFBO did not affect relaxations to endothelium-dependent vasodilators. BFBO concentration-dependently inhibited the cyclic GMP-hydrolyzing activity of basal PDE1, calmodulin-activated PDE1 and PDE5, and of cyclic AMP-hydrolyzing activity of PDE3 and PDE4 with IC_{50} values ranging from 40 to 130 μg/ml.
Conclusion: The butanol fraction of the aqueous methanol extract of *Berberis orthobotrys* roots induced pronounced endothelium-independent relaxations and inhibited contractile responses by acting directly at the vascular smooth muscle in the coronary artery. Moreover, BFBO potentiated relaxations induced by both cyclic GMP- and cyclic AMP-dependent vasodilators most likely due to its ability to inhibit several vascular PDEs, and in particular PDE4 and PDE5.

Abbreviations: ANP, atrial natriuretic peptide; BFBO, butanol fraction of *Berberis orthobotrys*; Cyclic GMP, cyclic guanosine 3′,5′-monophosphate; Cyclic AMP, cyclic adenosine 3′,5′-monophosphate; eNOS, endothelial nitric oxide synthase; PDE, phosphodiesterase; oxLDL, oxidized low density lipoprotein; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid; SNP, sodium nitroprusside; 1-EBIO, 1-ethyl-2-benzimidazolinone; L-NA, N-ω-nitro-L-arginine; EFBO, ethyl acetate fraction of *Berberis orthobotrys*; ARBO, aqueous residue of *Berberis orthobotrys*; IK_{Ca}, intermediate-conductance calcium-activated potassium channels.

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Introduction

Berberis is a large genus which belongs to the family Berberidaceae and includes over 400 species, such as *Berberis aquifolium* (Oregon grape), *Berberis aristata* (Indian barberry), *Berberis thunbergii* (red barberry), *Berberis vulgaris* (barberry) and *Berberis orthobotrys* (named "ishkeen" in Pakistan). This later plant is an indigenous shrub of Pakistan which is used by the people of the Gilgit region for the treatment of hypertension. Chemical studies of Berberis species have led to the identification of berberine, a yellow isoquinoline alkaloid, which has a long history of medicinal use in both Ayurvedic and Chinese medicine (Vuddanda et al., 2010). Several pharmacological properties have been previously reported for berberine, including antibacterial and anti-inflammatory activities, a beneficial effect on dyslipidemia and hyperglycemia, inhibition of cyclooxygenase-2, and improvement of neurological disorders (Kulkarni and Dhir, 2010). Berberine has also been shown to inhibit the generation of reactive oxygen species and the subsequent mitochondrial membrane potential collapse, as well as caspase-3 activation induced by oxLDL in endothelial cells (Hsieh et al., 2007). Moreover, berberine is a potent antineoplastic compound that inhibits cell proliferation through p53-dependent G1 arrest and p53-independent G2 arrest in HL-60 cells (Khan et al., 2010). Various clinical trials have evaluated its therapeutic properties in cardiovascular diseases (Derosa et al., 2012). Indeed, berberine treatment was shown to significantly decrease mortality in patients with congestive heart failure (Zeng et al., 2003). In addition, berbamine, another alkaloid extracted from *B. vulgaris*, has been shown to increase myocardial contractility by increasing myofilament Ca^{2+}-sensitivity via a protein kinase Cε-dependent signaling pathway (Zhang et al., 2011).

A recent study has shown that *B. orthobotrys* strongly decreased blood pressure in both normotensive and hypertensive rats (Alamgeer et al., 2013). Since previous studies have shown that extracts from plants used traditionally as antihypertensive medicine such *Parkia biglobosa*, cause potent endothelium-dependent relaxations involving NO of isolated arteries (Tokoudagba et al., 2010), the aim of the present study was to assess the potential of the *B. orthobotrys* extracts to affect vascular tone, to clarify the role of the endothelium and the underlying mechanism.

Materials and methods

Plant material and isolation

The roots of *B. orthobotrys* were collected from Shikiyote, Gilgit, Pakistan, in June 2011 and were identified by Dr. Shair Wali Khan, Assistant Professor of Botany, Karakuram International University, Pakistan. The voucher specimen No (BO-15-12) has been deposited in the Herbarium of the Faculty of Pharmacy, University of Sargodha, Pakistan. An aqueous methanol extract from *B. orthobotrys* was prepared by three successive macerations of 2 kg of roots in 5 l of a water–methanol mixture (70:30) for 72 h at room temperature followed by evaporation giving a drug extract ratio of 5:1. Thereafter, the methanol extract (100 g) was mixed with water (500 ml) and partitioned with the same volume of ethyl acetate (500 ml) for three successive times. The decantation and evaporation of the collected ethyl acetate layer provided 13 g of the ethyl acetate fraction of *B. orthobotrys* (EFBO). The remaining aqueous layer was further extracted with butanol (500 ml) for three successive times. The butanol layer was collected and evaporated providing 26.1 g of the butanolic fraction of *B. orthobotrys* (BFBO). The remaining aqueous layer was evaporated and provided 60.9 g of the aqueous residue of *B. orthobotrys* (ARBO). Each fraction was analyzed by thin-layer chromatography (TLC; Merck, Germany) eluted with n-butanol–acetic acid–water (4:1:1, v/v/v) and the presence of alkaloids was revealed using the Dragendorff 's reagent at 366 nm. All extracts were stored at 4°C before being used.

Chemicals and drugs

Levcromakalim, atrial natriuretic peptide (ANP) and ethylene glycol-bis(2-aminoethylether)-N,N,N',N' -tetraacetic acid (EGTA) were purchased from Tocris Bioscience (Bristol, UK). U46619 was supplied by Cayman Chemical (Ann Arbor, MI, USA), charybdotoxin (synthetic) and apamin by Latoxan (Valence, France), and bradykinin, sodium nitroprusside (SNP), forskolin, 1-EBIO, calcium ionophore A23187, N-ω-nitro-L-arginine (L-NA), isoproterenol hydrochloride, berberine hydrochloride by Sigma-Aldrich (Saint-Quentin Fallavier, France). All chemicals were of analytical grade.

HPLC profiles and quantification of berberine by LC/UV

Analytical HPLC analyses of the extracts fractions ARBO, BFBO and EFBO, and of the standards berberine and berbamine were performed on a LC-20 AD instrument system (Shimadzu) equipped with a SPD-M20A PDA detector, an evaporative light scattering detector (ELSD) serie 3300 (Alltech) and a LCMS-8030 detector (Shimadzu). For the ELSD, the N_2 flow was set at 2.5 l/min, and evaporation temperature was 60°C. The mobile phase consisted of 0.1% formic acid (solvent A) and MeOH + 0.1% formic acid (solvent B), and the flow was set to 0.4 ml/min. Separations were performed on an Uptisphere® Strategy C18-2 column (3.0 × 150 mm, 3 μm, Interchim) which was thermostatted at 40°C. For quantification of berberine in extracts, stock solutions extracts ARBO, BFBO and EFBO were prepared at concentrations of 8.1, 9.9 and 12.1 mg/ml, respectively. Each extract stock solution was further diluted two and four fold. For the standard of berberine, a stock solution of 1.01 mg/ml was prepared, from which five serial dilutions ranging from 202 to 25 μg/ml were obtained. For each sample, 10.0 μl was injected. The following gradient was used: 10% B isocratic for 4 min, gradient 4–20 min to 30% B, 20–30 min 30% B isocratic, 30–31 min to 100% B, 31–35 min 100% B isocratic, and 35–36 min to 10% B. The HPLC-UV-ELSD-MS chromatograms are shown in Fig. 1.

Quantification of berberine in the extracts was done by LC/UV at 345 nm and revealed the presence of 5.4%, 15% and 1.6% berberine in *Berberis* fractions ARBO, BFBO and EFBO respectively.

Vascular reactivity studies

Pig hearts were collected from the local slaughterhouse (Copvial, Holtzheim) and vascular reactivity was assessed as indicated previously (Ndiaye et al., 2003). Briefly, left circumflex coronary arteries were excised, cleaned of loose connective tissue and flushed with PBS without calcium to remove remaining blood. Rings of porcine coronary arteries (4–5 mm in length) were then suspended in organ baths containing oxygenated (95% O_2, 5% CO_2) Krebs bicarbonate solution (composition in mM: NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.18, $CaCl_2$ 1.25, $NaHCO_3$ 25 and D-glucose 11, pH 7.4, 37°C) for the determination of changes in isometric tension (basal tension 5 g). The integrity of the endothelium was checked with bradykinin (0.3 μM). For the assessment of the vasorelaxant properties of the extracts, rings were contracted (about 80% of the maximal contraction) with U46619 (a thromboxane A_2 receptor agonist) before construction of a concentration-relaxation curve to an extract. For assessment of the inhibition of contractile responses, rings were exposed to BFBO for 30 min before construction of a concentration-contraction curve either to KCl, U46619 or $CaCl_2$ in the presence of 40 mM KCl. In some experiments, rings were exposed to a low concentration of BFBO (10 μg/ml) for 30 min before contraction to U46619 and the subsequent construction of a concentration-dependent relaxation curve to an agonist.