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Antispasmodic and Ca^{++} antagonist potential of marrubiin, a labdane type diterpene from *Phlomis bracteosa*

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ABSTRACT

A tricyclic labdane type diterpene was isolated for the first time from ethyl acetate soluble part of *Phlomis bracteosa*. Its structure was confirmed by x-ray which was found to be marrubiin.When studied in isolated rabbit jejunum, marrubiin caused concentration-dependent relaxation of spontaneous and high K^+ (80 mM)-induced contractions, like that caused by verapamil, indicating that marrubiin exhibits spasmolytic activity, possibly mediated through Ca^{++} channel blocking action.

Key words: *Phlomis bracteosa*, labiatae, marrubiin, antispasmodic, Ca^{++} antagonist.

INTRODUCTION

The genus *Phlomis* belonging to family Labiatea consists of about 100 species in the world. [1, 2] Some *Phlomis* species are used as tonics and stimulants in Anatolian folk medicine. [3] Different *Phlomis* species are used in folk medicine as stimulant, tonic, analgesic, anti-diarrheal and considered useful for the treatment of ulcers, hemorrhoids and airways disorders. There are a few reports on the pharmacological activities, such as anti-inflammatory, immunosuppressive, anti mutagenic, anti-nociceptive, antifibrile, anti-oxidant, anti-malarial and anti-microbial effects. [4] The present work has led to the isolation of a known labdane type diterpene for the first time from *Phlomis bracteosa*, identified as marrubiin, which in pharmacological investigation revealed spasmolytic and Ca^{++} antagonistic effects.

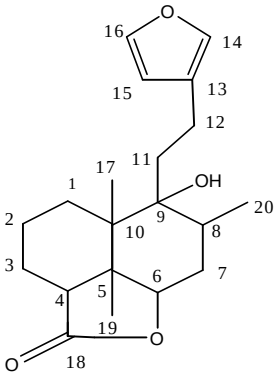

Figure 1. Structure of compound 1, marrubiin, a labdane type diterpene isolated from *Phlomis bracteosa*

MATERIALS AND METHODS

Plant material

Whole parts of *Phlomis bracteosa* were collected from Swat, NWFP, Pakistan in July 2005 and identified by Muhammad Naveed, Assistant Professor Botany Department University of Peshawar NWFP Pakistan. A Voucher Specimen was deposited in the Herbarium of the college.

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Extraction, Isolation and structure elucidation

The whole plant of *Phlomis bracteosa* was dried in shade, chopped and ground to coarse powder. The powdered plant (3 Kg) was initially extracted with methanol for 25 days at room temp. The combined methanolic extract was evaporated under reduced pressure giving residue (165 g). Using separating funnel, the methanol extract was partitioned applying different solvents i.e., hexane, chloroform, ethyl acetate, butanol and water (in order of increasing polarity) successively. [5] A portion of the ethyl acetate extract (70 g) was chromatographed over a silica gel column. The elution was carried out with a gradient of increasing polarity of hexane, ethyl acetate, and methanol. Fraction B was obtained through elution with n-hexane-ethyl acetate (1:1) and was subjected to column chromatography with the solvent system (n-hexane-ethyl acetate).Compound (**1**) was obtained from this fraction after eluting with *n*-hexane-ethyl acetate (3:7). Compound (**1**) was further purified by recrystallization from methanol. Column Chromatography (CC) was performed using silica gel, 70–230 mesh. Flash chromatography was carried out using silica gel 230–400 mesh. Thin layer chromatography (TLC) was performed with precoated silica gel G-25-UV_{254} plates and visualization was achieved using (UV, 254 nm) and by ceric sulphate in 10 % H_2SO_4 solution Silica gel (E. Merck, 230–400 mesh) was used for column chromatography. The IR and UV Spectra were recorded on a Jasco-320-A and Hitachi-UV-240 spectrophotometers, respectively. Optical rotations were measured on a Jasco-DIP-360-digital polarimeter using a 10-cm cell-tube. Mass spectra (EI- and HR-EI-MS) were measured in an electron impact mode on Finnigan MAT 12 or MAT 312 spectrophotometers; ions are presented in *m/z* (%). The ^{1}H-and ^{13}C-NMR spectra were recorded on a Bruker AMX-400 spectrometer in $CDCl_3$. The 2D-NMR spectra were recorded on Bruker AMX-400 spectrometer. Chemical shifts in parts per million (*ä*), relative to tetra methyl silane as an internal standard, and scalar coupling were reported in Hertz. The pulse conditions were as follows: for ^{1}H-NMR spectra, spectrometer frequency (SF) 400.032 MHz, acquisition time (AQ) 2.281 s, number of transients (NS) 128, receiver gain (RG) 812.7, temperature (TE) 300K, dwell time (DW) 69.6µs, per scan delay (DE)10µs, dummy scans (DS) 0, F_1 2431.15 Hz, F_2–1.77 Hz; for the ^{13}C-NMR spectra SF100.613 MHz, AQ 0.6 + 26 s, NS 25000, RG 16384, TE 300 K, DW 19.1µs, DE 20µs, DS2, F_1 23157.50 Hz; for the COSY 45° spectra SF 400.03 MHz, NS32, DS4, pulse (P1) 5.70 µs, P22.70 µs, TE 300 K, RG 267.4, DW 145.6µs, DE 10µs; for the NOESY experiments SF 300.133 MHz, NS 64, DE 305µs, pulse width (PW) 0.0, F1 2903.69 Hz, F_2 2903.69 Hz; for the HMQC spectra, SF 400.032 MHz, AQ 0.1491s, NS 32, DS 16, DE 10 µs, DW 145.6µs, RG 6502, TE 300 K, F_1 1688.52 Hz, F_2 3071.71 Hz; for the HMBC spectra SF 400.032 MHz, AQ 0.1491 s, RG 7296.2, NS 128, DW 145.6µs, DS 16, DE 10 µs, TE 300 K, F1 3212.30 Hz, F_2 23236.21 Hz.

X-rays analysis

The conditions for recording the spectra and the instruments have been described in (Sigitdinova et al., 1996).[6]

Chemicals and animals

Acetylcholine (ACh) and verapamil were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). The solvents, *n*-butanol, ethyl acetate, *n*-hexane, chloro form and methanol were from Merck (Darmstadt, Germany). Chemicals used for making physiological salt solutions were potassium chloride (Sigma Chemicals Co, St. Louis, MO, USA), calcium chloride, glucose, magnesium chloride, sodium bicarbonate, sodium dihydorgen phosphate (Merck, Darmstadt, Germany) and sodium chloride (BDH Laboratory supplies, Poole, England). All chemicals used were of the analytical grade. Rabbits (1.2-1.5 kg) of either sex or local breed were used in this study, housed at the Animal House of the Aga Khan University, maintained at 23-25°C and given a standard diet and tap water. Rabbits had free access to water, but food was withdrawn 24 hrs prior to experiment and killed by a blow on the back of the head. Experiments performed complied with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council [7] and were approved by the Ethical Committee of the Aga Khan University

Antispasmodic effect

The spasmolytic activity of the test compound was studied by using isolated rabbit jejunum as described previously. [8] Respective segments of 2-cm length were suspended in a 10 ml of Tyrode's solution and bubbled with carbogen gas at 37°C. The composition of the Tyrode's solution in mM was KCl 2.68, NaCl 136.9, $MgCl_2$ 1.05, $NaHCO_3$ 11.90, NaH_2PO_4 0.42, $CaCl_2$ 1.8, and glucose 5.55. A resting tension of 1 g was applied to each of the tissues and was kept constant throughout the experiment. Intestinal responses were recorded isotonically using a Bioscience transducer and Oscillograph. Each tissue was allowed to equilibrate for at least 30 min before the addition of any drug and then stabilized with a sub-maximal concentration of acetylcholine (0.3 µM) and the bath fluid was subsequently replaced with normal Tyrode solution before starting the experiment. Under these experimental conditions, rabbit jejunum exhibits spontaneous rhythmic contractions, allowing testing of the relaxant (spasmolytic) activity directly without the use of any agonist.

Determination of calcium antagonist activity

To assess whether the antispasmodic effect of the test compound is mediated through calcium channel blockade, high K^+ (80 mM) was used to depolarize the preparations as described by Farre et al.[9] . Addition of high K^+ to the tissue bath produced a sustained contraction. Relaxation of intestinal preparations by the compound, precontracted with K^+, was expressed as percent of the control response mediated by K^+.

Statistical analysis

The data expressed are mean ± standard error of mean (SEM) and analyzed by using GraphPad program (GraphPAD, San Diego, CA, USA).

RESULTS AND DISCUSSION

The ethyl acetate fraction obtained from methanolic extract of *Phlomis bracteosa* was subjected to silica gel column chromatography eluted with ethyl acetate hexane (8:2), afforded labdane type diterpene, compound **1** (Figure 1). It was isolated in white powder form and its molecular formula established as $C_{20}H_{28}O_4$ by x-rays and spectral techniques. By comparison its data with literature showed, that it is marrubiin.

When tested in isolated rabbit jejunum, marrubiin inhibited the spontaneous contractions with EC_{50} value of 1.3 µM (n=3), thus showing an antispasmodic effect (Figure 2A). Similarly verapamil, used as a positive control drug, relaxed the spontaneous contractions with EC_{50} values of 0.31 µM (n=3) as shown in Figure 2B. The contraction of smooth muscle preparations, including rabbit jejunum is dependent upon an increase in the cytoplasmic free [Ca^{++}], which activates the contractile elements. [10] The increase in intracellular Ca^{++} is due to either influx via voltage dependant L-type Ca^{++} channels (VDCs) or to release from intracellular stores in the sarcoplasmic reticulum. Periodic depolarization regulates the spontaneous movements of intestine and at the height of depolarization the action potential appears as a rapid influx of Ca^{++} via VDCs.[11] The inhibitory effect of the plant compound on spontaneous movements of jejunum.

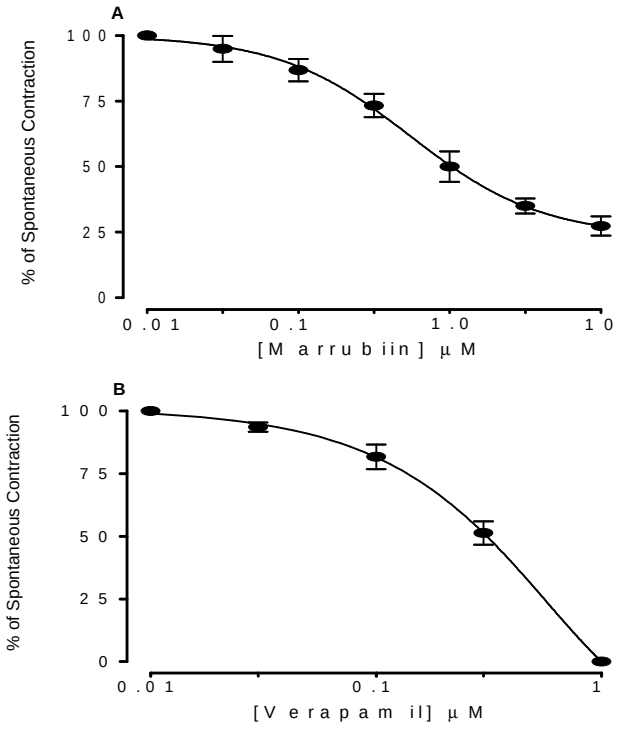

Figure 2. Concentration-dependent inhibitory effect of (A) marrubiin, isolated from *Phlomis bracteosa* and (B) verapamil on spontaneous contractions of isolated rabbit jejunum preparations. Values shown are mean ± SEM, n=3.

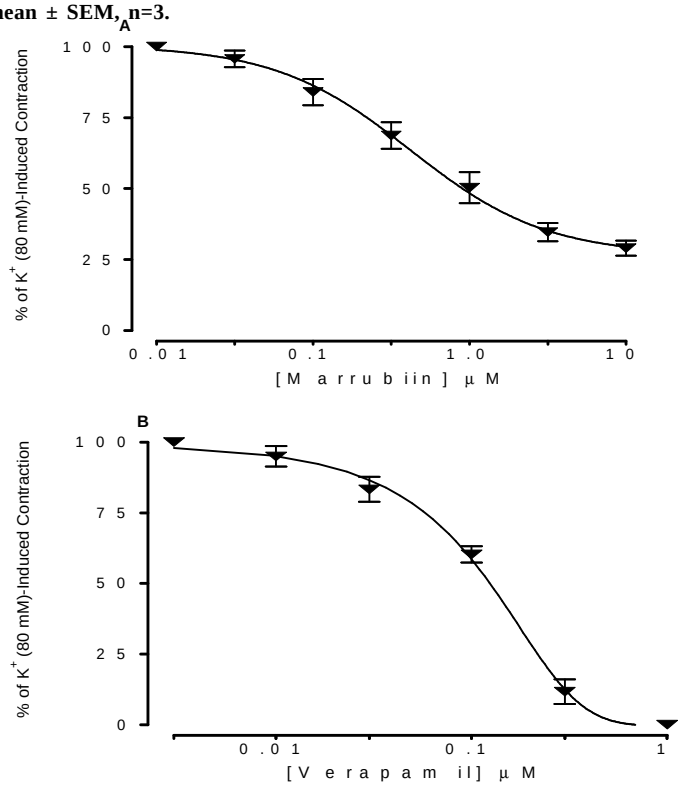

Figure 3. Concentration-dependent inhibitory effect of (A) marrubiin, isolated from *Phlomis bracteosa* and (B) verapamil on K^+-induced contractions of isolated rabbit jejunum preparations. Values shown are mean ± SEM, n=3.

may be due to interference either with the Ca^{++} release or with the Ca^{++} influx through VDCs. In our earlier studies, we have observed that the spasmolytic effect of the natural compounds is usually mediated through Ca^{++} channel blockade.[12-15] To see whether the spasmolytic effect of marrubiin is also mediated via the same mechanism, it was tested against high K^+ (80 mM)-induced contractions, which was relaxed by marrubiin with EC_{50} value of 1.4 µM (n=3) as shown in Figure 3A. At high concentration (> 30 mM), K^+ is known to cause smooth muscle contractions through opening of VDCs, thus allowing influx of extracellular Ca^{++} causing a contractile effect [16] and a substance causing inhibition of the high K^+-induced contraction is considered an inhibitor of the Ca^{++} influx.[17] Verapamil, a standard Ca^{++} antagonist[18] inhibited the high K^+ (80 mM)-induced contractions with EC_{50} values of 0.13 µM (n=3) as shown in Figure 3B. Thus the spasmolytic effect of the marrubiin may be visualized as the Ca^{++} channel blockade mechanism.

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